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Délivrance *in vivo* de siRNA et évaluation de leur effet antiviral contre le virus de la peste des petits ruminants (PPRV)

(*In vivo* delivery of siRNA and evaluation of its antiviral effect against peste des petits ruminants virus)

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LIST OF ABBREVIATIONS

Ad5	Adenovirus type 5
AGP	Anti-Genomic Promoter
BLI	Bioluminescent Imaging
DNA	Deoxy ribonucleic acid
CCD camera	Cooled Charged Couple Detector camera
$CCID_{50}$	Cell Culture Infectious Dose 50
CD150	Cluster of Differentiation (Cluster of Designation) 150, also called SLAM
CD46	Cluster of Differentiation (Cluster of Designation) 46, a complement regulatory protein
CDV	Canine Distemper Virus
CPE	Cytopathic Effect
CPPs	Cell Penetrating Peptides
DMV	Dolphin Morbillivirus
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, a neutral lipid
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane-chloride salt, a cationic lipid
ECTAD	Emergency Centre for Trans-boundary Animal Diseases (FAO)
EMEM	Eagle's Minimum Essential Medium
EMPRES	Emergency Preventative System (FAO)
FAO	Food and Agriculture Organization
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate
FMDV	Foot and Mouth Disease Virus
GFP	Green Fluorescent Protein
GP	Genomic Promoter
GREP	Global Rinderpest Eradication Program
IC/89	Wild type strain of peste des petits ruminants virus isolated from 1989 outbreak in Côte d'Ivoire (Ivory Coast)
IFN	Interferon
IFNAR-/-	Double knock-out mouse for Interferon type I receptor
Ig G	Immunoglobulin G

LOG	Logarithm base 10
MAB	Monoclonal Antibody
miRNA	micro RNA
MOI	Multiplicity of Infection
mRNA	messenger RNA
MV	Measles Virus
NPPRV	Nucleoprotein gene of peste des petits ruminants virus
OIE	Office International des Epizooties (The World Organisation for Animal Health)
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
pCMV	Plasmid containing the expression cassette driven by the human Cytomegalovirus Promoter
PCR	Polymerase Chain Reaction
PDV	Phocine Distemper Virus
PF6	PepFect6 (a cell penetrating peptide)
PF14	PepFect14 (a cell penetrating peptide)
PPR	Peste des Petits Ruminants
PPRV	Peste des Petits Ruminants Virus
PPRV 75/1	Vaccine strain of Peste des Petits Ruminants Virus obtained after attenuation of wildtype Nigeria 1975/1 strain
PTDs	Protein Transduction Domains
rAd_NPPRV1^{shRNA}	Recombinant replication deficient human Adenovirus type 5 expressing short hairpin RNAs against Nucleoprotein gene of Peste des Petits Ruminants Virus
rAd_SCR^{shRNA}	Recombinant replication deficient human Adenovirus type 5 expressing Scrambled short hairpin RNAs
rBac_EGFP^{shRNA}	Recombinant Baculovirus expressing an irrelevant short hairpin RNAs (against eGFP gene)
rBac_NPPRV1^{shRNA}	Recombinant Baculovirus expressing short hairpin RNAs against Nucleoprotein gene of Peste des Petits Ruminants Virus
RdRp	RNA-dependent RNA polymerase
RES	Reticulo-Endothelial System
RISC	RNA Induced Silencing Complex
RNA	Ribonucleic Acid

RNase	Ribonuclease; a nuclease that catalyzes the degradation of RNA
RNAi	RNA interference
RNP	Ribonucleoprotein
ROIs	Regions Of Interest
shRNA	short hairpin RNA
siRNA	small interfering RNA
SLAM	Signalling Lymphocyte Activation Molecule, found on activated T cells, B cells, thymocytes, macrophages and dendritic cells.
SRS	Suppressors of RNAi Silencing
TA	tibialis anterior muscle (of mouse)

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Délivrance *in vivo* de siRNA et évaluation de leur effet antiviral contre le virus de la peste des petits ruminants (PPRV)

RESUME EN FRANCAIS

par

NIZAMANI Zaheer Ahmed

La peste des petits ruminants (PPR) est une maladie virale contagieuse des chèvres, des moutons et de certains ruminants sauvages. Elle induit une maladie systémique sévère avec fièvre, dégradation de l'état général, troubles respiratoires et digestifs et aboutit très souvent à la mort de l'animal. La maladie est due à un morbillivirus de la famille des *Paramyxoviridae*. Les morbillivirus infectent de nombreuses espèces, l'homme par le virus de la rougeole les ruminants par les virus de la peste bovine et de la peste de petits ruminants, les carnivores par le virus de la maladie de Carré et les mammifères marins. Il s'agit de virus enveloppés pléiomorphes à ARN simple brin de polarité négative. Le virus est constitué de six protéines structurales, la nucléoprotéine étant la plus représentée.

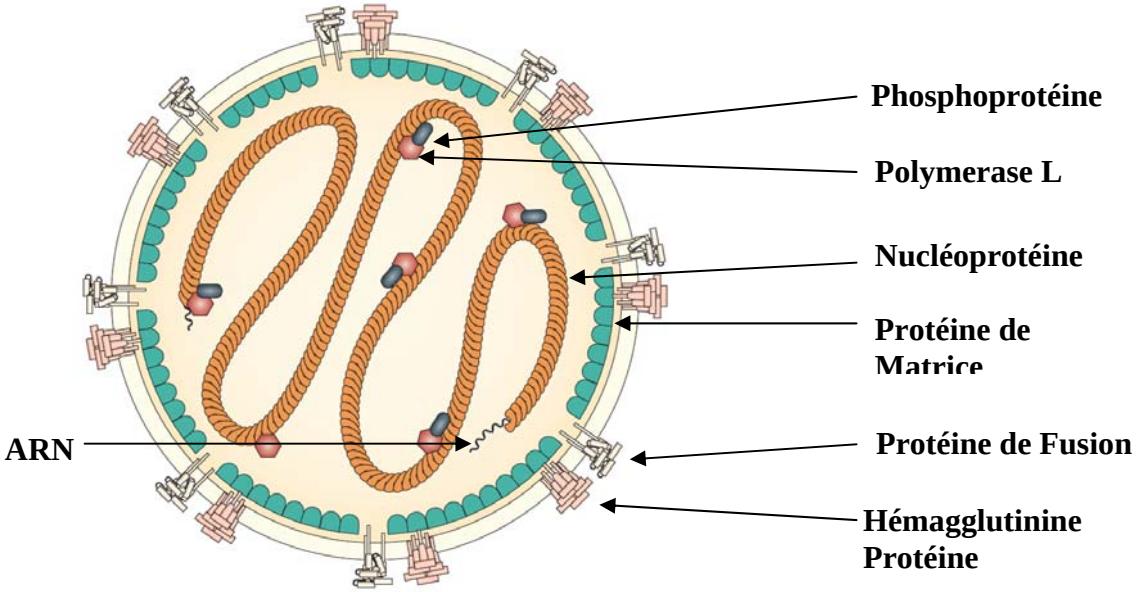


Figure 1 : Représentation schématique du Morbillivirus (d'après Moss et al., 2006 [1]).

Après pénétration dans l'organisme, le virus PPR infecte en premier lieu les cellules du système lymphoïde, puis une seconde phase de réplication au niveau des cellules épithéliales permet au virus d'être ré-excrété. Bien que peu résistant dans le milieu extérieur, le virus se transmet toutefois de façon efficace à la faveur de contacts directs entre animaux infectés et animaux sensibles.

Décrite pour la première fois en 1942 en Afrique de l'ouest, la maladie est désormais reconnue en Afrique, au Moyen-Orient et en Asie (Fig. 2). En Afrique, elle a longtemps été cantonnée à l'Afrique subsaharienne. Cependant, dans les cinq dernières années, elle a eu tendance à s'étendre vers le Maghreb (Maroc, 2008) et vers l'Afrique du Sud (Tanzanie, 2009).

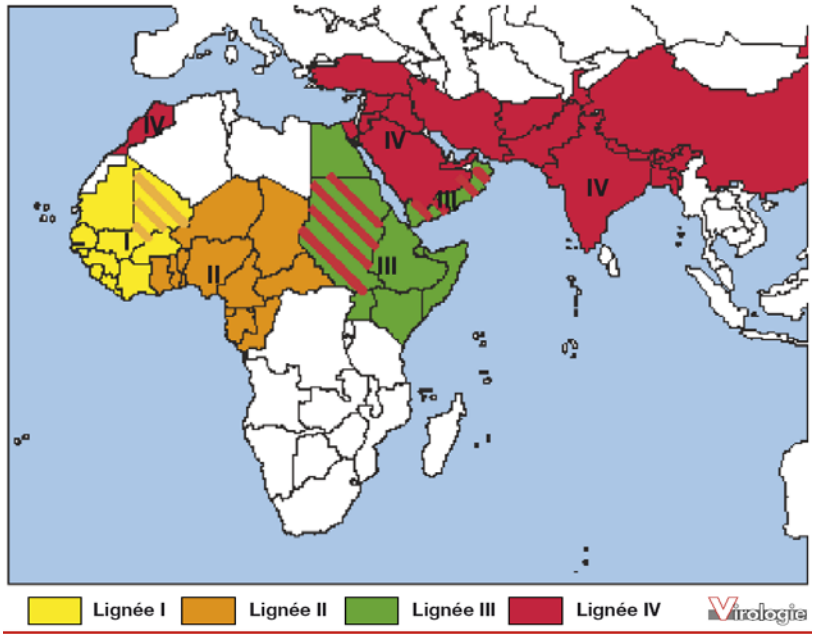


Figure 2: Distribution géographique des lignées phylogénétiques du virus de la PPR.

Parmi les moyens de lutte disponible, il y a un vaccin très efficace, permettant en une seule injection d'immuniser l'animal sur au moins 3 ans. Toutefois, ce vaccin est en pratique peu utilisé dans les zones où la maladie sévit de façon enzootique. La vaccination est pratiquement toujours mise en œuvre en situation d'urgence, lorsque l'incidence clinique est déjà très marquée. Le contrôle de la maladie est alors plus complexe, plus long et plus couteux. La possibilité de combiner une thérapie antivirale avec la vaccination pourrait, le cas échéant, permettre d'accélérer le contrôle de la maladie.

Parmi les différentes stratégies thérapeutiques antivirales, il en est une qui suscite actuellement et depuis dix ans, des recherches actives. Il s'agit de l'interférence ARN. L'interférence ARN est un mécanisme naturel des cellules eucaryotes qui permet la régulation de l'expression de gènes, qu'ils soient du soi ou du non soi (d'origine virale). Elle est basée sur l'interaction d'un simple brin d'ARN d'une vingtaine de nucléotides (small interfering RNA, siRNA) avec un ARN messager présentant la séquence complémentaire du siRNA. Cette interaction médiée par un complexe protéique appelé RISC pour RNA « induced silencing complex » permet la dégradation spécifique de l'ARNm cible. Cette régulation post-

transcriptionnelle est parfois si spécifique de la séquence cible qu'une seule mutation dans le siRNA peut annuler l'effet. Toutefois, toute mutation n'implique pas forcément une perte d'activité. Cela dépend pour l'essentiel de la position de la mutation dans le siRNA, certaines positions étant critiques pour l'effet interférent. Par ailleurs, une interaction partielle entre un siRNA et un autre ARNm distinct de sa cible est toujours possible et peut aboutir à une dérégulation de l'expression d'une protéine importante avec des conséquences négatives. Tous ces éléments permettent d'expliquer les contraintes liées à l'utilisation de l'interférence ARN en thérapie antivirale :

- le risque d'échappement du virus aux siRNA par simple mutation, soit dans le site de reconnaissance, soit à distance de ce site mais à un endroit qui entraîne un changement de conformation de l'ARNm rendant le site inaccessible ;
- le risque d'effets indésirables par interférence ARN sur des ARNm non ciblés ;
- le risque d'effets secondaires liés à la compétition des siRNA thérapeutiques avec les siRNA endogènes ayant un rôle dans la régulation du métabolisme cellulaire

A ces contraintes, s'ajoute la difficulté de délivrer efficacement les siRNA dans le cytoplasme cellulaire, près du noyau où se localise le complexe protéique responsable de l'interférence ARN. *In vitro*, la délivrance est assez aisée avec des agents de transfection basés sur des liposomes, des peptides ou des vecteurs viraux. *In vivo*, la mise en œuvre des mêmes systèmes de délivrance aboutit le plus souvent à une perte d'efficacité. Différentes raisons peuvent expliquer cet écart. L'exposition des siRNA aux enzymes circulantes dont les RNases, est une première cause de dégradation rapide des molécules actives, avant même qu'elles n'aient la capacité d'entrer dans le cytoplasme des cellules qu'elles sont censées traiter. Les vecteurs viraux peuvent avoir des limites dans le ciblage des cellules et par ailleurs, ils posent des questions sur le plan de leur innocuité par rapport à leur capacité réplicative le cas échéant ou parce qu'ils ont une phase nucléaire dans l'expression des siRNA.

Tout ceci illustre le challenge auquel sont confrontés les chercheurs pour que les ARN interférents parviennent jusqu'à exploitation thérapeutique. Seuls quelques uns d'entre eux, délivrés localement au niveau des muqueuses, ont été jusqu'à des essais en phase clinique.

Le CIRAD a engagé des travaux sur l'interférence ARN en 2004. Le premier objectif consistait à identifier des siRNA actifs *in vitro* sur plusieurs morbillivirus. Le gène codant la

nucléoprotéine (N) virale a été initialement choisie car les outils de détection de ce gène ou de son produit étaient disponibles (sondes, anticorps monoclonaux, etc.). Des régions conservées de ce gène ont été identifiées et soumises à sélection de séquences siRNA actives par un logiciel commercial ou selon des critères extraits de la littérature. Aucun siRNA ciblant une région suffisamment conservée du gène N pour être actif sur plusieurs morbillivirus n'a pu être identifié. En revanche, trois sites ont été identifiés qui peuvent être efficacement ciblés *in vitro* par des siRNA spécifiques de trois morbillivirus différents, délivrés soit par transfection soit par un adénovirus recombinant exprimant des ARN interférents. La stratégie actuelle du laboratoire consiste à explorer la capacité d'échappement des morbillivirus au contrôle des siRNA et à traiter la question de la délivrance *in vivo* des siRNA. Le premier volet est l'objet d'un autre travail de thèse alors que le second volet est au cœur de ce mémoire.

Pour délivrer un siRNA actif contre le virus de la PPR, notre travail a commencé par la production et l'évaluation *in vivo* de deux systèmes de délivrance, d'une part, des liposomes et d'autre part, un adénovirus recombinant exprimant des siRNA. L'adénovirus que nous avons choisi était le même que ceux utilisés par des groupes chinois ayant réussi à interférer avec la réplication de virus porcins dans l'espèce cible. Nous avons donc décidé d'adopter un protocole très similaire, adapté à la chèvre. Toutefois, ni les liposomes, ni l'adénovirus ne se sont révélés suffisamment efficaces. Face à ce résultat plutôt décevant, nous avons décidé d'explorer d'autres systèmes de délivrance et en parallèle de développer un modèle petit animal permettant d'évaluer *in vivo* et comparativement, les différents systèmes de délivrance de siRNA. Deux systèmes alternatifs de délivrance ont été développés puis évalués *in vitro*. Le premier d'entre eux est un peptide capable de pénétrer la membrane cellulaire. Le second est un vecteur baculovirus adapté à l'expression en cellules de mammifères. Ces deux systèmes ont été évalués *in vitro* en comparaison avec l'adénovirus précédemment développé, dans l'attente de les tester *in vivo*. Dans le but de comparer quantitativement des effets interférents chez l'animal tout en limitant le nombre d'animaux utilisés pour des raisons éthiques et en réduisant les coûts, nous avons opté pour le développement d'un modèle souris avec évaluation de l'interférence ARN par imagerie *in vivo*.

1. Evaluation de deux systèmes de délivrance de siRNA chez la chèvre

Différentes formulations de liposomes basées sur un mélange de liposome cationique 1,2-dioleoyl-3-trimethylammonium-propane-chloride (DOTAP), de lipide neutre 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) et de la cardiolipine anionique ont été préparées. Ces préparations ont ensuite été intimement mixées avec le siRNA en phase acqueuse pour

former des complexes incorporant le siRNA. Un adénovirus recombinant exprimant un court ARN en forme de tête d'épingle à cheveux (*short hairpin* RNA ou shRNA) avait été produit en utilisant un kit commercial, juste avant que ne commence ce travail de thèse.

Les lipocomplexes ont été mis au contact des cellules pendant cinq heures, avec une concentration finale de siRNA équivalente à 100 nM. D'autres cellules ont été transduites avec l'adénovirus recombinant à une multiplicité d'infection de 80. Vingt quatre heures après contact avec les lipocomplexes ou 72 heures après transduction par l'adénovirus, les cellules ont été infectées avec le virus PPR à une multiplicité d'infection de 0,1 dose cytopathique infectieuse 50% (DCI_{50}) par cellule.

L'interférence virale a été mesurée sur le développement de l'effet cytopathique produit par le virus PPR ou par quantification de la nucléoprotéine virale par cytométrie en flux. Le contrôle positif correspondait à un ARN interférent transfecté par la Lipofectamine™ 2000 (Invitrogen) et le contrôle négatif était un ARN interférant sans rapport avec le virus PPR. Une formulation lipidique utilisée avec un rapport de concentrations de 5 pour 1 siRNA s'est révélée efficace pour neutraliser 80% de l'effet cytopathique dû au virus PPR. Cet effet était comparable à celui obtenu avec la Lipofectamine™ 2000. En revanche, la formulation que nous avons sélectionnée conservait un effet inhibiteur à 45% en présence de 60% de sérum de chèvre, ce qui se rapproche de l'environnement normal dans lequel le siRNA devra évoluer une fois administré à l'animal. Le bas coût de production de cette formulation permet aussi son application à grande échelle. La Lipofectamine™ 2000 ou d'autres liposomes disponibles dans le commerce seraient efficaces en présence de fortes concentrations de sérum comme indiqués par leurs fournisseurs. Cependant, le coût astronomique de leur administration chez la chèvre ne permettait pas d'envisager cette solution. L'adénovirus pour sa part, a inhibé l'effet cytopathique viral à environ 95% et il n'est pas sensible à la présence de sérum dans le milieu. La meilleure formulation lipidique et l'adénovirus ont donc été utilisés chez la chèvre pour tenter d'inhiber la réplication du virus PPR.

Trente chèvres ont été réparties en trois groupes. Un premier groupe a reçu l'adénovirus recombinant exprimant le siRNA d'intérêt (rAd_NPPRV1^{shRNA}) à la dose de $0{,}5x10^{10}$ DCI_{50} par voie intraveineuse. Un second groupe a reçu la même dose d'un adénovirus du commerce exprimant un siRNA sans rapport avec le virus PPR (rAd_SCR^{shRNA}). Enfin, le troisième groupe a reçu trois administrations consécutives à 24 heures d'intervalle de 12 mg de siRNA incorporés dans des lipoplexes. Tous les animaux ont été éprouvés avec une souche virulente

de virus PPR, 48 heures après la première administration des antiviraux. L'examen quotidien des animaux selon une grille de scores cliniques a permis de quantifier le développement de la maladie lorsqu'est présente. Les différences observées, en faveur de la formulation lipidique, n'ont pas été significatives au plan statistique. Ceci souligne la difficulté de délivrer correctement des siRNA dans les cellules cibles chez l'animal.

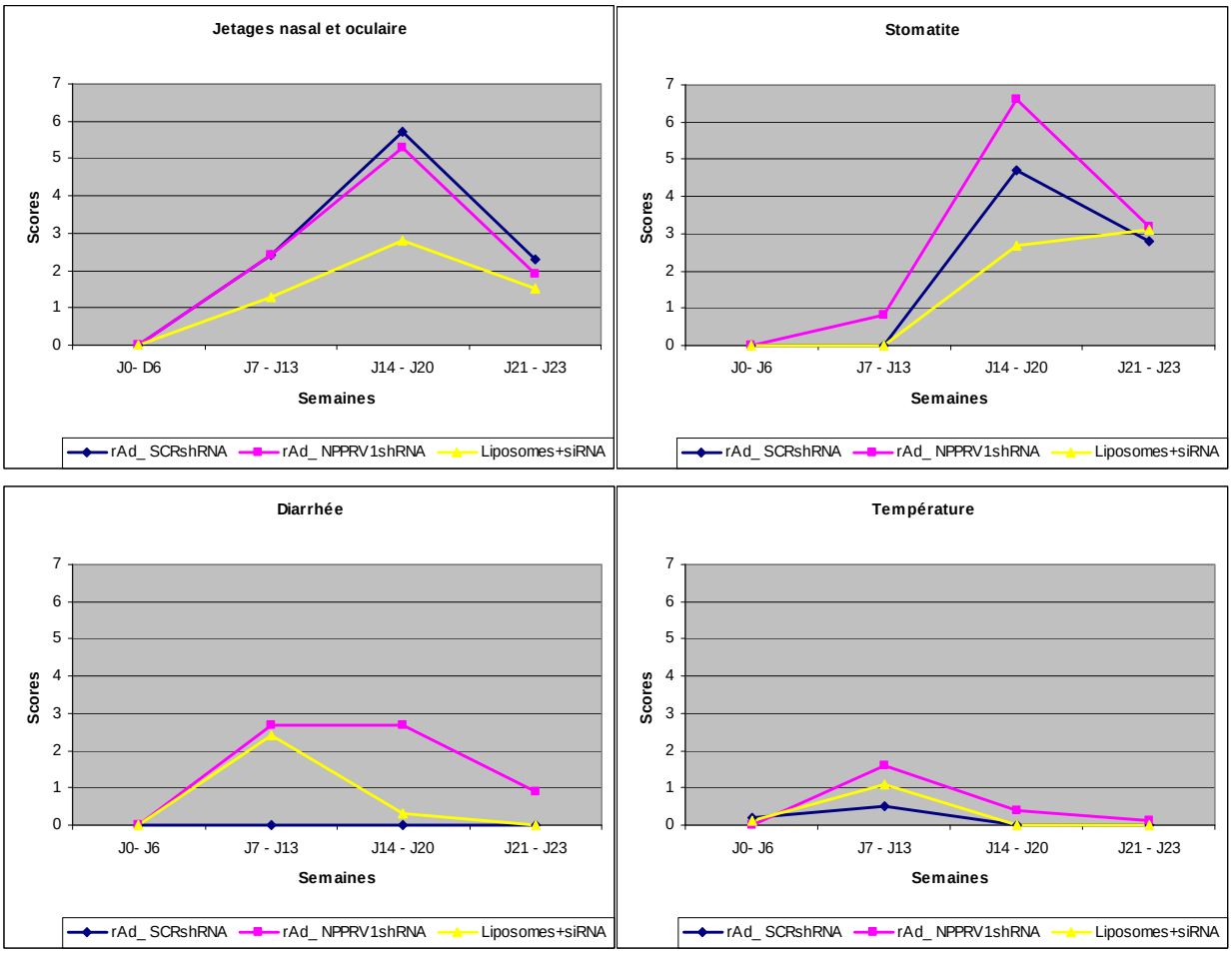


Figure 3 : Evolution des écoulements oculaires et nasaux, stomatite, diarrhée et températures après infection PPR dans les groupes de chèvres traitées avec liposome+siRNA NPPRV, rAd_NPPRV1^{shRNA} et rAd_SCR^{shRNA}.

Ces premiers résultats insuffisants nous ont amenés à travailler dans deux directions parallèles mais complémentaires. Nous avons cherché d'une part, à développer d'autres systèmes de délivrances plus performantes et d'autre part, à mettre au point un modèle d'évaluation comparative des différents systèmes de délivrance chez la souris, plus compatible avec les exigences éthiques en matière d'expérimentation animale.

2. Evaluation *in vitro* de deux nouveaux systèmes de délivrance

Au cours de la thèse, une collaboration nouée avec un groupe allemand (Institut Friedrich Loeffer) et un autre groupe suédois (Université de Stockholm) a permis d'obtenir deux nouveaux systèmes de délivrance de siRNA à potentiel *in vivo*. Le premier est un baculovirus BacMam adapté à l'expression en cellules de mammifères et dans lequel le groupe allemand a inséré la même cassette d'expression de shRNA que nous avions clonée dans l'adénovirus (rBac_NPPRV1^{shRNA}). La représentation schématique de ce nouveau vecteur en comparaison avec l'adénovirus est reproduite en Figure 4. Nous avons également obtenu du même groupe un baculovirus exprimant un autre shRNA sans rapport avec la PPR (shRNA contre la protéine eGFP).

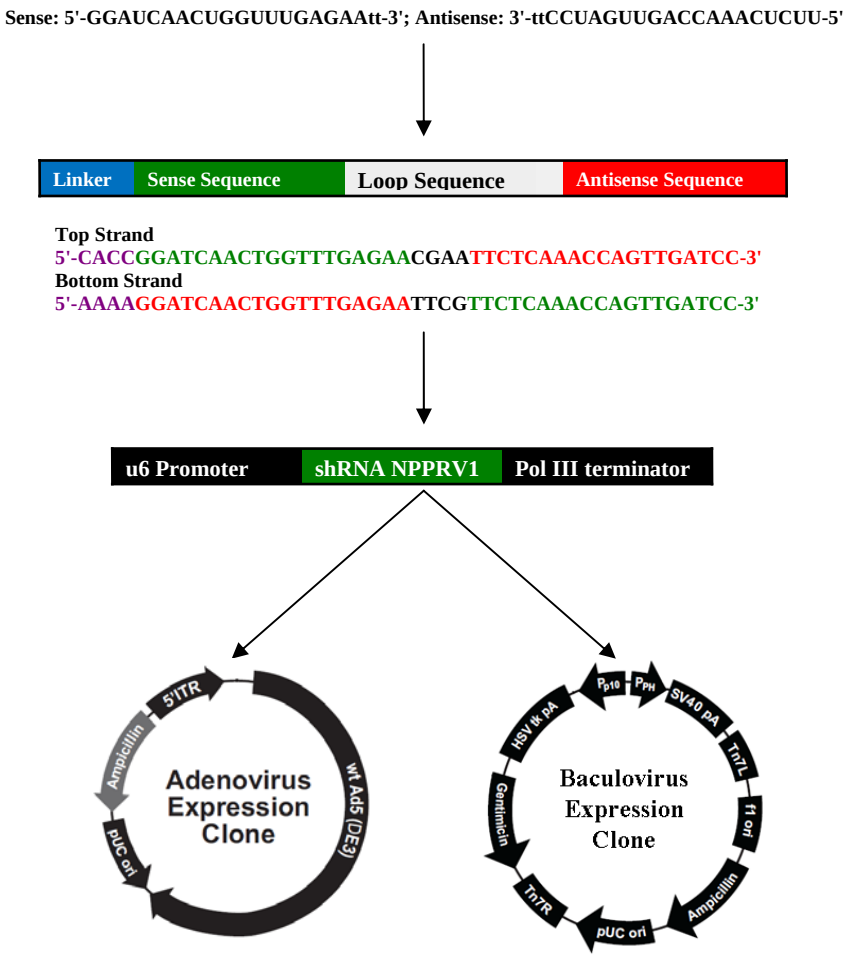


Figure 4 : Représentation schématique des constructions de vecteurs viraux.

Deux peptides nommés PF6 et PF14 nous ont été fournis par le groupe suédois pour une évaluation *in vitro*. Ces molécules, définies comme CPPs (pour *cell penetrating peptides*) modifiées chimiquement, sont capables de former des complexes stables non-covalents avec le siRNA et de délivrer efficacement dans l'intérieur de la cellule. Ces peptides avaient les séquences suivantes:

	Sequences peptidiques
PepFect 6	Stearyl-AGYLLGK[$KK_2sa_4qn_4$]INLKALAALAKKIL-NH_2
PepFect 14	Stearyl-LLOOLAAAALOOLL -NH_2

Les peptides sont complexés avec le siRNA à température ambiante pendant une heure, des ratio siRNA/peptide de 1/5 à 1/15 ont été évalués.

Les deux vecteurs viraux et les peptides ont été comparés *in vitro* à différentes doses avec l'agent de transfection classique Lipofectamine™ 2000 pour délivrer le même siRNA contre le virus PPR. Une première comparaison entre les deux vecteurs viraux a montré une meilleure efficacité *in vitro* du baculovirus aux fortes concentrations (Fig. 5). L'adénovirus a au maximum entraîné une réduction de la progénie virale de 0,7 Log DCI_{50}, alors que le baculovirus atteignait 2,2 Log DCI_{50} et la Lipofectamine™ 2000 3 à 4 Log DCI_{50}.

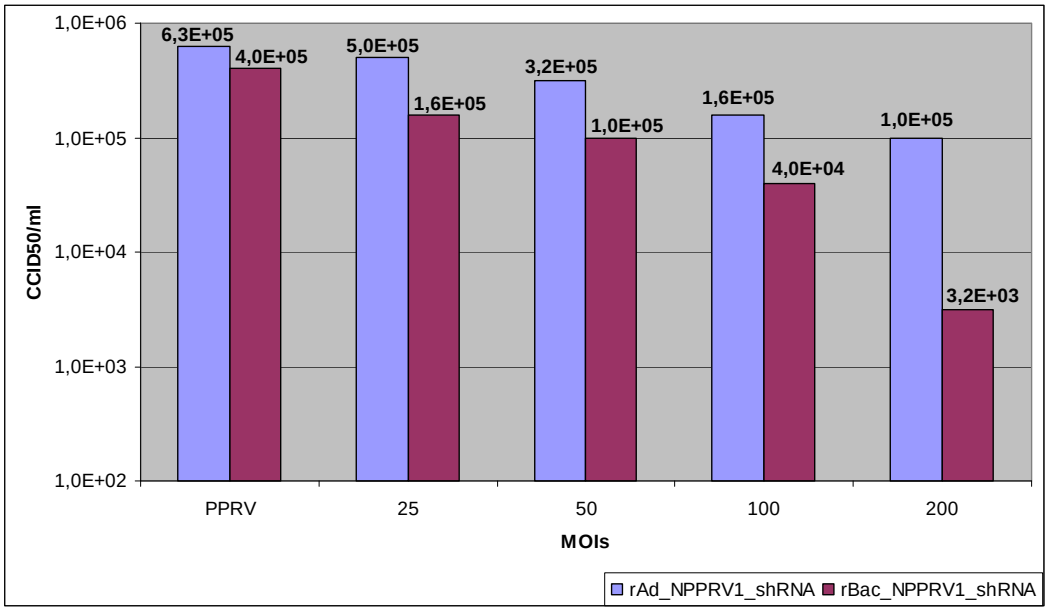


Figure 5 : Inhibition de la production du virus PPR par l'adénovirus ou le baculovirus recombinant exprimant le shRNA NPPRV1 (rBac_NPPRV1^{shRNA}**)**. Le titrage du virus PPR a été effectué 96 heures après infection à MOI 0,01.

Les deux peptides se sont avérés efficaces dans la délivrance de siRNA *in vitro*. Le PF6 a été le plus efficace, permettant d'inhiber l'expression de la nucléoprotéine virale jusqu'à 99% comparativement à 74% avec la lipofectamine (Fig. 6). Cependant, le PF14 a été le plus résistant à l'effet délétère de sérum de fœtus de bovin jusqu'à concentration de 30%. L'addition de ce sérum en forte concentration avait pour objectif de tester la résistance des peptides en milieu riche en sérum comme ce sera le cas lorsqu'ils seront délivrés *in vivo*.

Toutefois, même si le PF6 a une activité fortement réduite par le sérum, à la concentration de 30% dans le milieu de culture, il reste encore plus efficace que le PF14. Les résultats de cette étude ont montré que le baculovirus recombinant et les peptides PF6 et PF14 pouvait présenter un intérêt dans la délivrance siRNA. Il serait alors intéressant de les évaluer *in vivo*, sur un modèle souris.

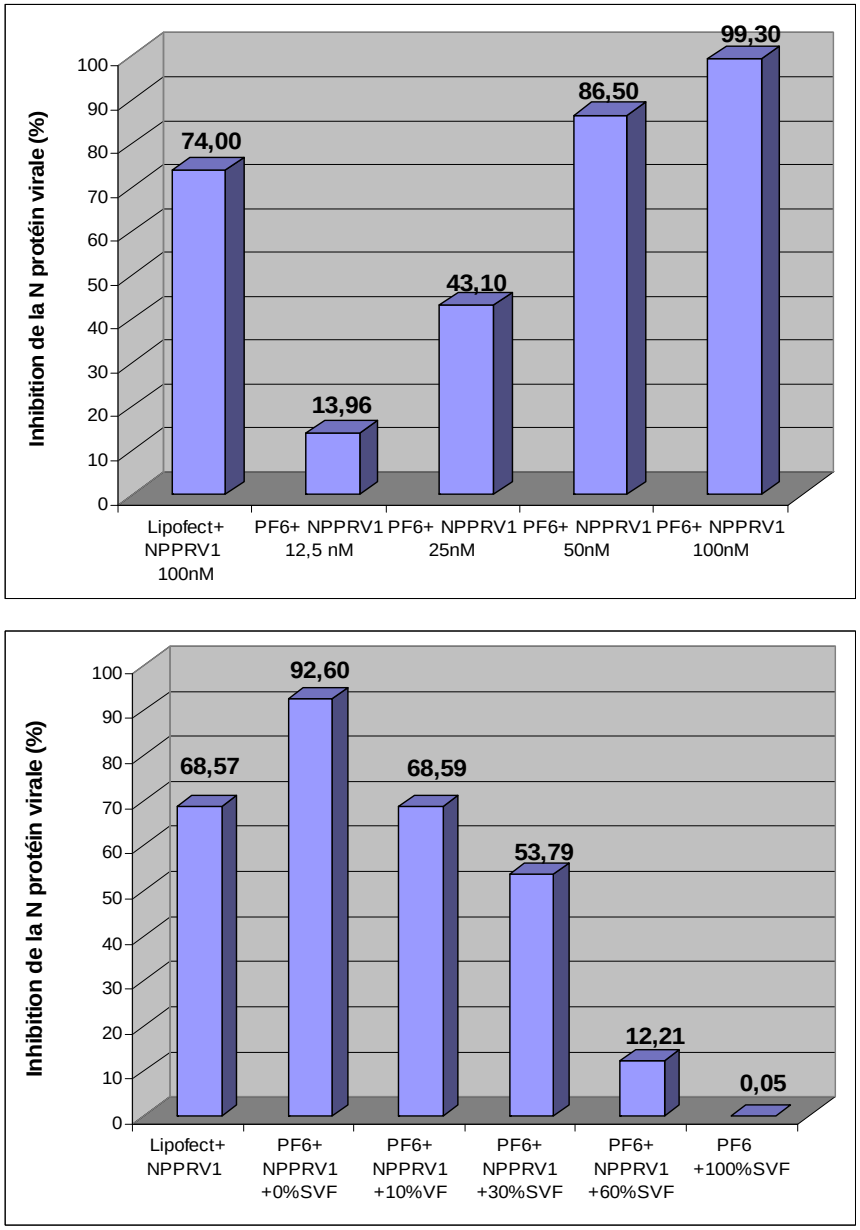


Figure 6 : Effet de différentes concentrations de siRNA complexées avec le PF6 (graphe du haut) et impact de la présence de sérum de fœtus de bovin dans les cultures sur l'efficacité du PF6 (graphe du bas).

3. Contribution au développement d'un modèle petit animal pour l'évaluation *in vivo* de systèmes de délivrance de siRNA

Face à la difficulté de passer des études *in vitro* à l'évaluation *in vivo* des siRNA dans l'espèce cible qui concerne ce travail (les petits ruminants, voir section 1), nous avons envisagé la possibilité de développer un modèle souris pour comparer différents systèmes de délivrance. Toutefois, les souris classiques de laboratoire ne sont pas sensibles au virus de la PPR. Des souris délétées du gène exprimant le récepteur aux interférons de type I se sont révélées sensibles à l'infection par le virus de la fièvre catarrhale ovine due à un orbivirus. Cependant, quand nous avons testés ces souris avec le virus PPR, aucune infection n'a pu être mise en évidence par voie intra-péritonéale. Avec un autre morbillivirus, le virus de la rougeole, une équipe INSERM de Lyon a pu développer un modèle d'infection sur des souris exprimant le récepteur du virus de la rougeole, croisées avec des souris délétées du gène exprimant le récepteur aux interférons de type I. Le laboratoire a donc programmé de tester ces souris avec le virus de la PPR et le cas échéant de préparer une lignée de souris transgénique exprimant le récepteur du virus PPR. Toutefois, les délais pour aboutir n'étaient pas compatibles avec la durée de cette thèse. Aussi, un modèle intermédiaire a été défini. Il s'agissait de préparer un système d'expression basé sur un ARNm comprenant la séquence cible du siRNA_NPPRV1 et un gène rapporteur. Ce système d'expression doit être inhibé par le siRNA *in vitro*. Puis il sera injecté chez l'animal et on tentera alors d'inhiber ce système par la délivrance *in vivo* du siRNA. Afin de réduire le nombre d'animaux utilisé pour ce travail, nous avons choisi d'utiliser l'imagerie *in vivo* qui permet de suivre de façon cinétique chez le même animal l'effet interférent. Les gènes rapporteurs utilisables en imagerie *in vivo* sont les gènes codant pour des enzymes de luminescence, car elles permettent de mesurer un signal dans la profondeur des tissus.

Une première construction a été faite avec la séquence siRNA_NPPRV1 placée en amont du gène de la Firefly luciférase (Fig. 7). Cette construction a été validée *in vitro*, dans le sens où le siRNA_NPPRV1 a été capable d'éteindre la luminescence de la Firefly (Tab. 1). Pour passer cette construction *in vivo*, il nous a fallu identifier un site d'expression périphérique, circonscrit mais accessible aux siRNA par le sang périphérique. Notre choix s'est porté sur une injection intramusculaire dans le muscle tibial antérieur. Après avoir validé le principe dans un essai réduit sur souris, nous avons engagé un essai pour tester la délivrance de nos siRNA avec une préparation à base de liposomes fournie par une entreprise privée avec laquelle nous avons noué un partenariat. Cette entreprise avait démontré précédemment dans un autre modèle l'efficacité de son système de délivrance de siRNA chez la souris.

Cependant, cet essai n'a pas permis de mettre en évidence un effet interférent, principalement à cause d'une hétérogénéité d'expression importante d'un membre à l'autre d'un même animal et entre animaux. Jusqu'à 30-40% des souris peuvent ne pas répondre à l'injection dans le muscle tibialis. Et pour celles qui répondent, le niveau d'expression peut varier dans des proportions importantes. Cette hétérogénéité est propre à la qualité de l'injection dans le muscle tibialis. Pour parer à cette variabilité, nous avons décidé de combiner au plasmide rapporteur cible du siRNA_NPPRV1, un autre rapporteur qui lui est insensible. Ainsi, il nous sera possible de normaliser le signal d'une souris à l'autre.

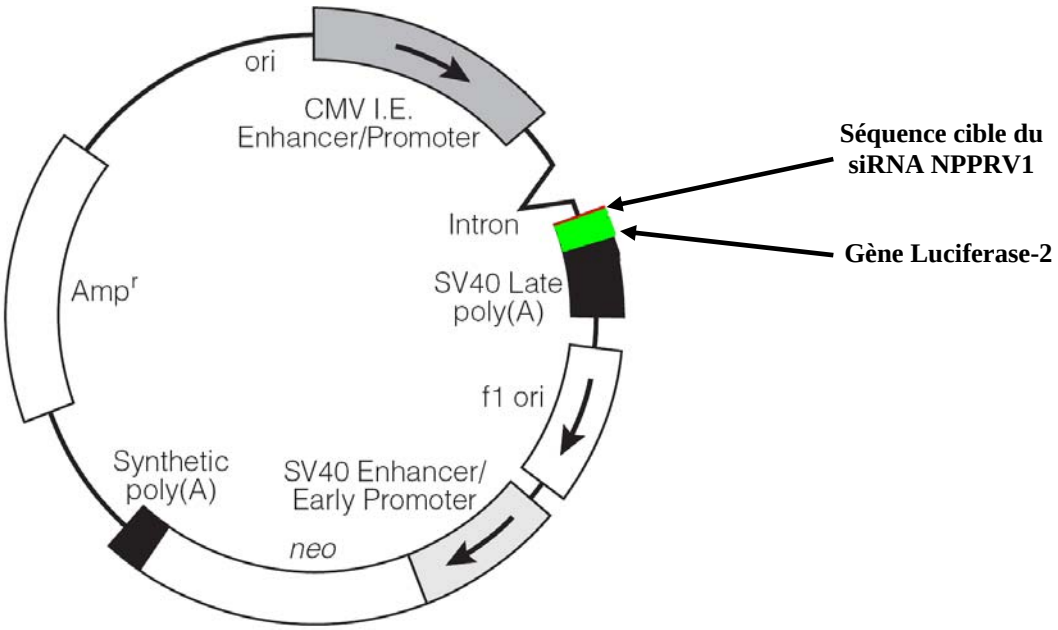


Figure 7 : Schéma de la construction préparée pour l'imagerie *in vivo*. La séquence cible du siRNA_NPPRV1 est placée directement en amont de la séquence ARNm du gène de la Firefly luciférase. L'expression est contrôlée par le promoteur du cytomégalovirus humain (pCMV).

Tableau 1 : Validation de la construction du gène rapporteur placé en aval de la séquence cible du siRNA. Les résultats sont exprimés en unités de luminescence relatives, le contrôle positif étant le plasmide d'origine du gène de la luciférine (pGL4.51) et le contrôle négatif étant les cellules non transfectées.

	pGL4.51 (100 ng)		psiRNA-Fluc (100 ng)		Contrôle négatif	
	24h	48h	24h	48h	24h	48h
20 pmole siRNA NPPRV1	16924	3183	13672	2584	134	174
20 pmole -siRNA IR*	26527	7440	634592	92659		
40 pmole siRNA NPPRV1	17954	6166	9592	2344		
40 pmole-siRNA IR	21335	3047	257299	72207		

*IR: irrelevant

Après une revue exhaustive de la littérature, notre choix pour le deuxième gène rapporteur, tenant compte de nos contraintes (deuxième marqueur luminescence, n'interférant pas avec la mesure de la Firefly luciférase), s'est porté sur la Rénilla luciférase. Un plasmide commercial a été acquis et évalué *in vitro* puis *in vivo*. Nous avons pu alors montrer que la normalisation pouvait être effectuée (Fig. 8).

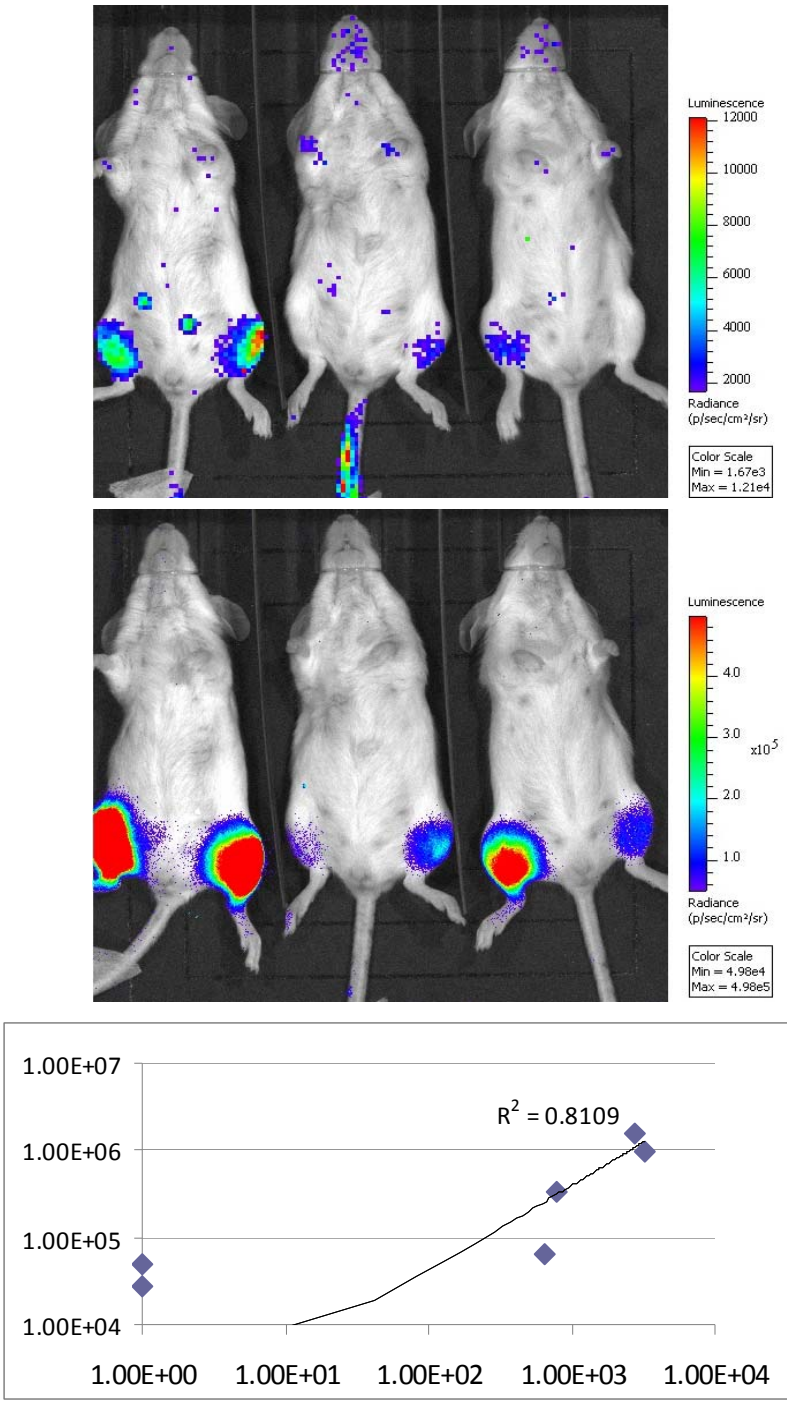


Figure 8 : Mesure de la co-expression des gènes Renilla (en haut) et siRNA_NPPRV1-Firefly luciférase (en bas). Les plasmides d'expression ont été mélangés puis injectés dans le muscle tibialis. On constate une bonne corrélation des deux signaux lorsqu'il y a expression (partie droite du graphe représentant en abscisse le niveau d'expression Renilla et en ordonnée, le niveau d'expression Firefly).

Cet essai nous incite désormais à faire une construction plasmidique unique contenant les deux cassettes d'expression de sorte à éliminer tout risque d'expression différentielle entre les deux plasmides mélangés. Une fois cette construction faite et validée *in vivo*, nous serons en mesure de mettre en œuvre un nouvel essai de délivrance de siRNA *in vivo*.

4. Discussion générale et conclusion

La délivrance *in vivo* des siRNA est le point critique pour franchir l'espace qui sépare l'identification d'une séquence active à un produit thérapeutique. Les quelques publications décrivant des essais réussis sur gros animaux sont rares. Chez l'homme, il y a moins de 6 essais cliniques en phase II pour une délivrance systémique de siRNA qui sont actuellement engagées. Lorsque nous avons tentés de délivrer un siRNA *in vivo* chez la chèvre, en se basant sur les données de la littérature, nous n'avons pas obtenu d'effet significatif. Cela est du probablement à l'incapacité de nos systèmes de délivrance à protéger le siRNA de sa dégradation ou de sa dispersion dans l'organisme, ou son efficacité insuffisante dans l'adressage du siRNA dans le cytoplasme des cellules cibles. Toutes ces hypothèses soulignent la nécessité de continuer à travailler sur la qualité de systèmes de délivrance et de développer un outil d'évaluation *in vivo* de ces systèmes de délivrance.

Dans cette thèse, nous avons développé deux nouveaux systèmes de délivrance qui se sont avérés *in vitro* plus efficaces que ceux que nous avions testés précédemment sur la chèvre. Par ailleurs, un partenariat noué avec le secteur privé nous a permis d'avoir accès un troisième système de délivrance, également prometteur dans notre approche puisqu'ayant déjà fait ses preuves chez la souris dans un autre modèle. Les trois systèmes de délivrance sont des candidats jugés très intéressants pour une approche *in vivo*.

Plusieurs raisons nous ont amené à penser à un modèle souris pour tester ces systèmes de délivrance. En premier lieu, l'administration de siRNA sur l'espèce cible, le petit ruminant est très couteux lorsqu'il s'agit d'administrer du siRNA synthétisé chimiquement (de l'ordre de 1.700 euros les 3 milligrammes de siRNA). Sur la souris, les quantités sont bien évidemment considérablement réduites (40 µg/souris). Par ailleurs, l'évaluation de l'efficacité de la délivrance chez l'espèce cible ne peut se faire que par épreuve virulente avec le virus PPR ce qui pose des questions d'éthique lorsqu'il s'agit de sacrifier un grand nombre d'animaux. En outre, la souris est un animal facile à imager, ce qui permettait d'envisager un modèle dynamique de suivi de l'activité interférente chez le même animal, permettant à nouveau de réduire le nombre d'animaux nécessaires à l'expérience. Enfin, nous avons opté pour le

développement d'un modèle non infectieux chez la souris permettant d'éviter à l'animal la souffrance liée au développement d'une maladie systémique dans le but de comparer différents systèmes de délivrance. Cette première étape sera cependant suivie d'une seconde toujours chez la souris afin d'évaluer le meilleur système de délivrance dans le contexte d'une épreuve infectieuse. Toutefois, les essais d'épreuve virulente sur souris seront limités à quelques systèmes de délivrance, les moins bons ayant été écartés en phase 1. Ce modèle d'épreuve sur souris nécessite la production d'animaux transgéniques sensibles au virus PPR. Il est en cours d'acquisition au laboratoire. Il sera un intermédiaire indispensable avant passage sur chèvres.

Nos travaux ont permis de montrer que le modèle non infectieux d'évaluation des systèmes de délivrance était à portée de main. Il ne reste plus qu'à établir la reproductibilité de ce modèle pour ensuite commencer l'évaluation de nos trois systèmes de délivrance.

En conclusion, après avoir été confronté en direct à des difficultés de mise en œuvre de la délivrance de siRNA anti PPR chez la chèvre, nous avons engagé un travail de fond pour identifier de nouveaux systèmes de délivrance qui s'avèrent très prometteurs, basé soit sur un vecteur viral, soit sur un peptide, soit sur une préparation à base de liposomes, couvrant ainsi assez largement les systèmes déjà testés avec succès dans d'autres modèles d'atténuation de l'expression de gènes *in vivo*. Par ailleurs, nos efforts pour le développement d'un modèle *in vivo* ont été fructueux, dans un domaine où les compétences du laboratoire étaient inexistantes au début de cette thèse. L'état d'avancement de ce projet, même s'il n'a pas permis d'arriver à montrer une efficacité *in vivo* de notre siRNA, offre désormais des perspectives de résultats à court terme. C'est la contribution majeure de ce travail.

INTRODUCTION

Peste des petits ruminants (PPR) is an infectious and highly contagious viral disease of domestic and wild small ruminants, clinically characterized by pyrexia, pneumonia, oral

erosive lesions, occulo-nasal discharges, and diarrhoea. Morbidity rate of 90% and mortality rate of 50–80% may occur in susceptible populations. The disease transmission occurs primarily through the aerosols from infected animals in close contact with healthy animals. It is caused by the virus of *Peste des Petits Ruminants* (PPRV), which belongs to the genus *Morbillivirus* and family *Paramyxoviridae*. Goats and sheep are the primary targets of the virus, however, gazelles, ibex and deer are also susceptible to the disease [2]. Furthermore, unapparent infections in cattle, buffalo, and camels have been reported [3-5].

PPR is an emerging trans-boundary disease, which has apparently spread from its original place of discovery in West Africa (Côte d'Ivoire) to East Africa, Middle East, and South Asia [6-10]. PPR is presently extending its traditionally boundaries and has reached Tibet and Tajikistan on one hand and on the other hand has recently emerged in Turkey, Morocco and Tunis, thus threatening Europe [11-15]. It is on the list of animal diseases that must be declared, in case of an outbreak, to the World Organization for Animal Health (Office international des epizooties, OIE). The economic losses are due to mortality, loss of productivity in sick animals, treatment costs and ban on the international trade once the disease is declared.

Morbilliviruses are important pathogens of humans, ruminants, carnivores and marine mammals characterized by their high contagiosity and severity of the disease. Good vaccines inducing long-term immunity are available against measles, rinderpest (RP), peste des petits ruminants (PPR) and canine distemper (CD). However, the vaccination coverage is only partial and recurrent outbreaks of measles, CD and PPR are observed. No specific treatment is available when the disease appears and serious and definitive after-effects can develop particularly of the nervous system [16, 17]. Therefore, an antiviral therapy could be useful not only in the treatment, control and eradication strategies against PPR, but it will also have implications for diseases caused by other morbilliviruses.

Since its first discovery in 1998, the phenomenon of RNA interference (RNAi) has been successfully used against many viruses *in vitro,* both through chemical and viral vectors. RNAi can be used as an effective mean for antiviral therapy if well delivered into the cells. However, efforts for *in vivo* delivery have been facing similar obstacles as gene therapy since 1980s, i.e., low bioavailability, non-specificity, toxicity, low transfection efficiency of chemical vectors in presence of serum, and immunogenicity of viral vectors.

With the aim to develop an antiviral strategy against these diseases, various siRNA sequences were designed at CIRAD, to target the nucleoprotein (N) gene of peste des petits ruminants

virus (PPRV), rinderpest virus (RPV) and measles virus (MV) which could effectively inhibit viral replication, N protein expression and copies of viral genomes *in vitro*. However, therapeutic application of siRNA requires correct delivery of these molecules to the cell cytoplasm which poses significant problems *in vivo*. To overcome these problems several delivery strategies have been developed, like those based on chemical vectors transfecting siRNAs (small interfering RNAs) as well as those based on viral vectors expressing nucleic acids [18]. Although siRNAs have been successfully delivered against viral infections by local administration [19], the systemic delivery of siRNAs is still a major stumbling block for the use of siRNAs to therapeutic use. The siRNAs have been successfully delivered systemically through chemical vectors against hepatitis B virus (HBV) [20], hepatitis C virus (HCV) [21], and HIV [22], but these involved the murine models. Whereas viral vectors have been used to inhibit infections of farm animals with some success. Adenoviral vector expressing shRNA against FMDV has been used in swine [23], while recombinant herpesvirus of turkey (HVT) [24] and avian leukosis virus-based retroviral vectors [25] targeting the Marek's disease virus (MDV) have been able to moderately reduce viremia in chicken. Thus far, no work has been reported on *in vivo* delivery of siRNAs against morbilliviruses.

The present work is divided in two parts. The first part concerns the review of literature about PPR, RNAi, and vectors used for siRNA delivery. The second part concerns the results obtained in the course of the research project. This second part is divided in three sections and ended by a general discussion.

REVIEW OF THE LITERATURE

1. Peste de Petites Ruminants (PPR): A Morbillivirus infection

Peste des petits ruminants virus (PPRV) shares many similarities with the diseases caused by other viruses in the *Morbillivirus* genus, not only in terms of structure but also in its pathogenesis and epidemiology. Therefore, first of all, there will be a general review about morbillivirus classification, economic importance, epidemiology, and structure while in the later part will be reviewed the PPR itself.

1.1 Classification

The morbilliviruses make up an antigenically homogenous group in the family *Paramyxoviridae*. The viruses belonging to the *Morbillivirus* genus are negative sense, single stranded, non segmented RNA viruses which are classified into the order *Mononegavirales*, family *Paramyxoviridae* and sub-family *Paramyxovirinae*. In addition to PPRV, the *Morbillivirus* genus includes measles virus (MV), dolphin and porpoise morbilliviruses (DMV & PMV), canine distemper virus (CDV), phocine distemper virus (PDV), and rinderpest virus (RPV). Morbilliviruses can infect a wide variety of hosts like primates, ruminants, carnivores and marine mammals with devastating ecological, demographic and economic consequences. In general, each morbillivirus infects only one order of mammals to cause serious disease (Fig. 1), however, these viruses can extend host range by jumping the species barrier. Thus RPV infects artiodactyls, PPRV infects sheep and goats, MV infects primates, CDV infects young canines, felines, and phoques, and two new members of *Morbillivirus* genus, the DMV and PMV affect dolphins and porpoises, respectively. Moreover, each of the morbilliviruses can be also further classified into several distinct phylogenetic lineages.

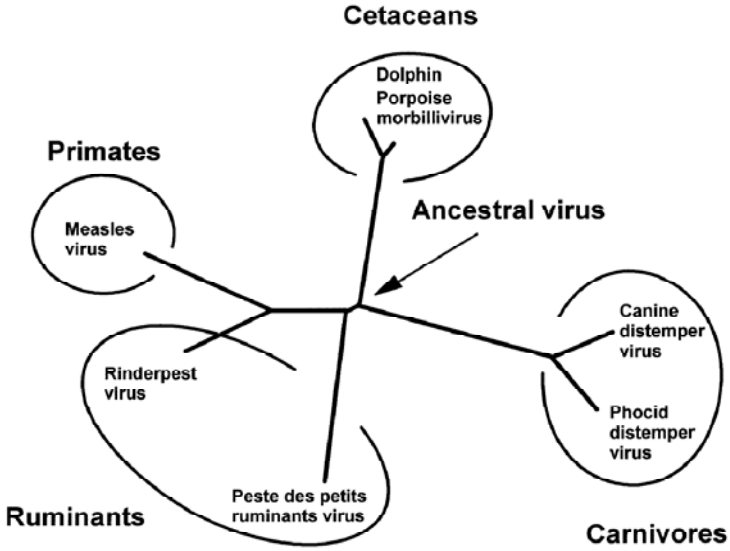


Figure 1: Phylogenetic tree showing the relationships between the different morbilliviruses based on partial sequence of the phosphoprotein (P) gene, according to Barrett *et al*., 2006 [26].

1.2 Morbillivirus infections: Geographical distribution, epidemiology and economic impact

Morbilliviruses share many similarities. They are primarily lymphotrophic and secondarily epitheliotropic. They use CD150 or signaling lymphocyte activation molecule (SLAM), found on lymphocytes, monocytes, and dendritic cells, as a primary receptor [27]. Each virus in the *Morbillivirus* group has a single serotype, and the individuals surviving infection generally develop long lived immunity involving cell mediated immune response [28]. However, despite being antigenically conserved, they are genetically variable and have mutation rates similar to other RNA viruses [29]. There is no carrier state; therefore all morbilliviral infections require a constant supply of susceptible hosts for maintenance continuity of chain of transmission for the infection. Through mathematical models it has been estimated that at least a susceptible population of 250,000-500,000 is needed to MV and RPV maintain MV and RPV infections [30-32].

Morbilliviral infections have plagued human and animal populations since centuries. Among the group, rinderpest (RP), also called cattle plague, is the oldest, best documented, and most dreaded disease of farm animals, with descriptions of the disease in Europe dating back to late Roman times [33]. The disease originating in Asia spread to Europe, Africa towards the end of nineteenth century, Brazil in 1920 and Australia in 1923 [34]. Being highly infectious and with about 90% mortality rate, it caused terrible destruction of cattle, affecting agriculture and rural livelihoods bringing famine and starvation. Outbreaks of RP in Europe in 1920s lead to creation of OIE in 1932 [35]. The disease in Europe was controlled by early twentieth century through zoo-sanitary measures except for few introductions due to importation of infected cattle and zoo animals [36]. However efforts were made to develop a vaccine, and it was not until 1962 that a safe tissue culture derived attenuated vaccine was developed [37]. Despite various attempts at controlling the disease with vaccinations, the disease resurged many times [36]. In 1994, Food and Agriculture Organization (FAO) launched "Global Rinderpest Eradication Programme (GREP)" in association with World Organization for Animal Health (OIE), and donor agencies, for control and eradication of rinderpest through vaccination campaigns. The programs of vaccination followed by sero-monitoring were very successful and last outbreak of RP was reported in 2001. The vaccination has been stopped since 2007 and FAO is in the process of ending the field operations. An international declaration of Global Rinderpest Freedom is expected to be made by 2011. After smallpox in humans, this would only be the second time in history that a disease has been eradicated worldwide [38].

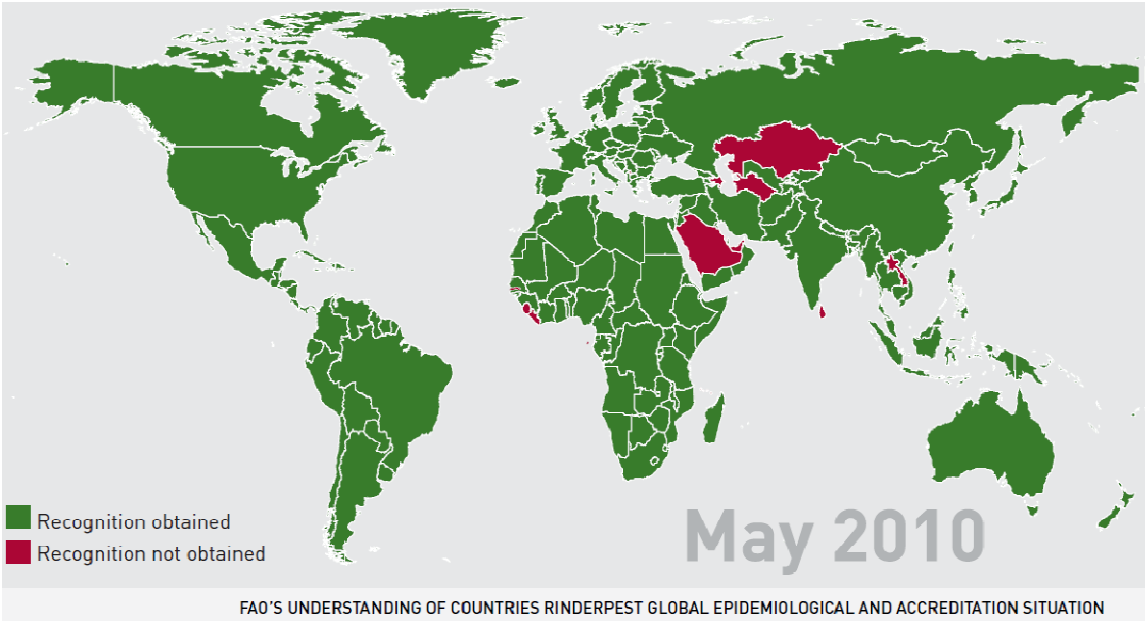


Figure 2: The Global Rinderpest Eradication Programme. Status report on progress made to May 2010 in the eradication of rinderpest: highlighting success stories and actions required prior to Global Declaration in 2011. According to FAO [38].

RPV has been suggested to be the morbillivirus archetype [39]. Measles virus is closely related to the RPV, therefore it is assumed that MV probably evolved in an environment where cattle and humans lived in proximity [26, 40] and that may have been after a start of livestock farming in the early centers of human civilization in the Middle East [41]. However, using phylogenic techniques based on sequences of haemagglutinin (H) and neuraminidase (N) genes, it has been estimated that divergence between MV and RPV occurred more recently around 11th and 12th centuries [41]. Despite availability of an effective vaccine since 1963, measles outbreaks still occur in poorly vaccinated areas of Asia, Africa, and even Europe and more than 30 million cases of acute measles with approximately 345,000 cases of infant deaths are annually reported [42]. "European Region strategic plan 2005-2010" of WHO, which included a target for eliminating measles, has not been achieved as measles cases have been reported even in March 2010 with an outbreak in Ireland and Slovenia [43]. South Africa has also experienced a large measles outbreak with 17,354 laboratory-confirmed measles cases diagnosed during the period January 2009 to August 2010 and despite a nationwide mass measles vaccination campaign new cases continue to be confirmed throughout the country [44]. Even in the months of August and September 2010, cases been reported in ProMED-mail (International Society for Infectious Diseases) for measles in India, Zimbabwe, and Brazil [45-47].

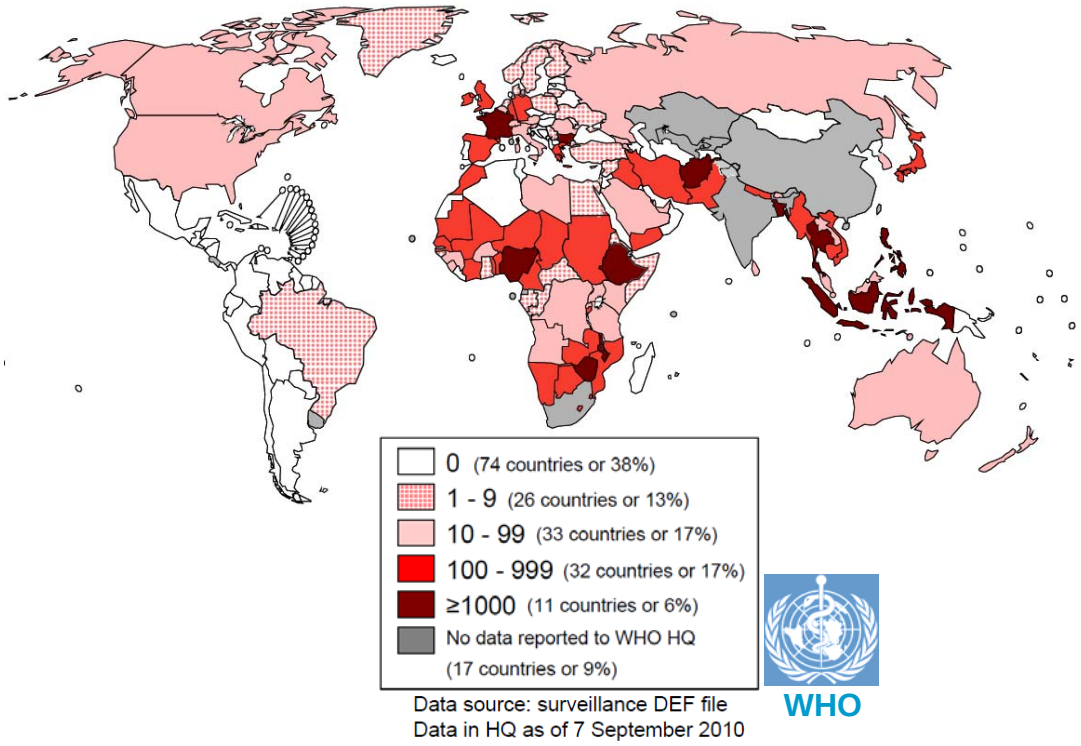


Figure 3: Number of reported measles cases with onset date from Feb 2010 to Sept 2010 [44]. The number of reported cases of measles reflects a small proportion of the true number of cases occurring in the community. Many measles cases do not seek health care or, if diagnosed, are not reported thus the data shown in the map under-represents the true number of cases, particularly those occurring in the last one to two months.

Canine distemper (CD) is a fatal disease for many domestic as well as wild carnivores with an ability to cross species barrier. It has a global distribution with seven phylogenetic lineages found in Arctic, America, Europe, Asia, and Africa [48]. CD shares some antigenic and taxonomic similarities with measles virus, but it is assumed to have emerged much more recently [29]. Effective vaccines are available but resurgences of canine distemper have been reported even in vaccinated dogs [49, 50]. The haemagglutinin (H) protein of the virus interacts with host cell receptors for morbillivirus attachment and this protein of CD has a higher mutation rate as compared to mumps and measles [29]. This partly explains a broader host range for CDV as compared to other morbilliviruses. Furthermore, after mapping of the distribution of mutations sites on H protein of CDV isolates from new host species, it has been found these are not only to be under influence of positive selection but also that the majority of amino acid substitutions fall into the SLAM-binding domains of the H protein, which are responsible for host specificity [51, 52].

PPR was first described by Gargadennec & Lalanne in 1942, as an outbreak in Côte d'Ivoire, they reported it to be a highly fatal disease and resembling rinderpest but affecting only sheep and goats while cattle remained unaffected [6]. PPR was later found in Senegal in 1962 [53].

Although PPR is a recently recognized disease, the disease probably already existed but diagnosis was perhaps confused with rinderpest and pasteurellosis [54]. Since, small ruminants are less susceptible to even more virulent strains of RPV and the infection tends to be either sub-clinical or mild thus perhaps even some of the reported RP outbreaks in small ruminants in past may have been due to PPRV [55]. Mornet *et al*., had reported in 1956 that virulent strains of PPRV was not pathogenic for cattle but conferred immunity against RP [56]. Based on these results the authors suggested that PPRV can be considered a variant of RPV, better adapted to the small ruminants. Serologic cross-reactions with RPV also confused early recognition of the disease as a distinct entity of separate etiology, however by late 1970, experimental infections, virological studies, and serologic studies established that PPR was a disease distinct from RP [57-59]. The two viruses were definitively differentiated by biochemical tests as well in 1987 [60]. Until 1979 this disease was declared to be limited to West and central Africa [61], however it was reported in 1984 that apparent RP outbreaks in 1972 and 1973 in Sudan were later serologically confirmed as PPR [7]. By 1990s, it was also reported in East Africa in Kenya [8] and Uganda and Ethiopia [62]. Outside Africa, the PPR was first discovered in southern India in 1987 [9] and later on it spread across Arabian Peninsula, Middle East and the remaining subcontinent by 1995 [10]. PPR still continues extending towards areas that were not previously endemic, and it was declared in Tibet, China in 2007 [11] and Morocco in 2008 [15] and has also been reported in Tunis [14].

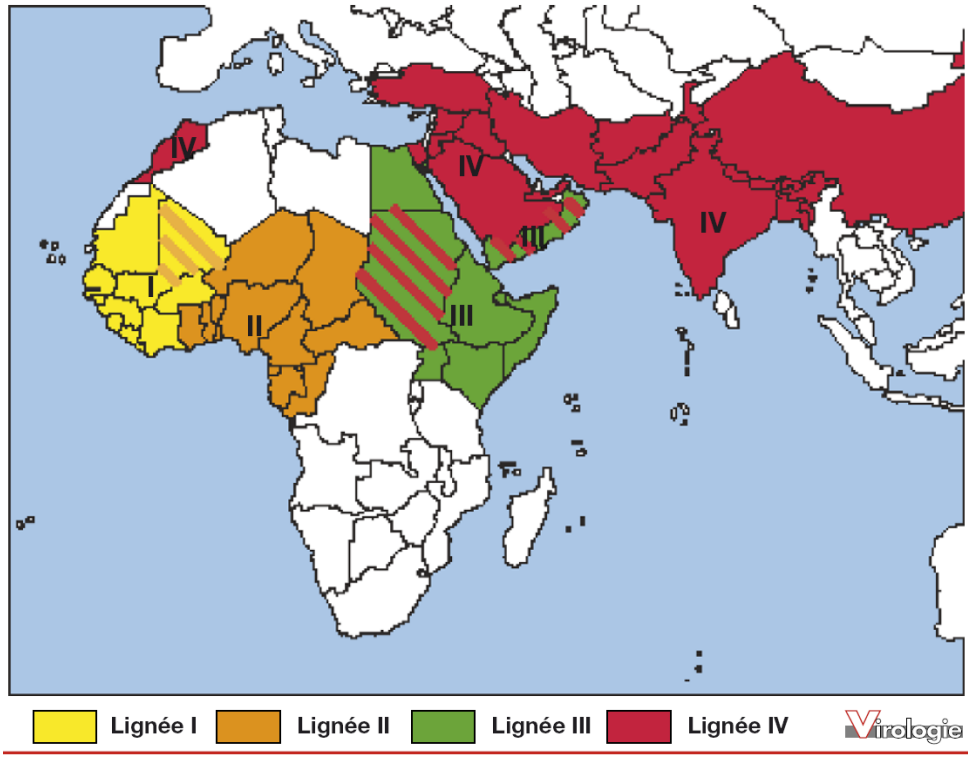


Figure 4: Geographical distribution of the phylogenetic lineages of PPRV. [63]

PPRV found in different geographic areas has been differentiated into four (1–4) lineages based upon nucleic acid sequencing (Fig. 4). The apparent rapid spread of PPR during the last three decades is perhaps due to a combination of factors like greater trade of live animals, wider availability better diagnostic tools, vigilance and more awareness after eradication of RP [64]. Furthermore, it has also been postulated that the chronological spread of PPR from West Africa to Bangladesh may give a false impression that the disease spread has occurred from West to East and that perhaps PPRV like RPV also has its origin in Eurasia and has spread towards the West [37].

The economical impact of PPR is greatest on the poorest populations. Since large ruminants are costly, a large proportion of rural populations breed sheep and goats for subsistence. The small ruminants are therefore considered to be the "cattle of the poor". Not only are the goats and sheep raised as a source of meat and milk for family consumption, but they are also an important source of income. In an international study aimed at prioritizing research on animal diseases in order to have maximum impact on improving conditions in developing countries, highlighted the economic importance of goats and sheep for the poor and ranked them at first or second position for Asia and Africa [65]. Furthermore, the PPR was ranked among the top ten diseases in these animals and thus affecting livelihoods of the poor in Asia and Africa. It thus showed a need for the control of PPR to be taken into consideration for any poverty alleviation policies [65]. PPR is one of the priorities of FAO Emergency Preventative System (EMPRES) program which considers more than a billion small ruminants to be at risk of PPRV infection and the rate of global domestic small ruminant population to be at risk was 62.5% [66, 67]. A socioeconomic study by Emergency Centre for Trans-boundary Animal Diseases (ECTAD) of the Food and Agriculture Organization (FAO) of the United Nations, conducted to understand the livelihood impacts of PPR in the arid and semi-arid lands of Northern Kenya, found that PPR outbreaks resulted in better-off farmers slipping into poverty and the poor and very poor became destitute [68]. The livestock-derived income losses due to PPR ranged between 21% and 99 % and it was found that most households were unable to maintain sustainable flock size owing to high mortality. Therefore, they had to leave pastoralist livelihoods resulting in a long term dependency on food aid and a drain on national resources [68]. Eradication of rinderpest has increased the relative economic importance, thus highlighting the need for PPR control.

1.3 Morbillivirus genome, structure and replication

The PPRV shares structural, biological, antigenic and molecular features in common with other morbilliviruses. Morbilliviruses are enveloped, pleomorphic, single stranded, negative sense RNA viruses. When viewed by electron microscopy, the members of *Paramyxoviridae* family are indistinguishable [69]. The virion of RPV attains a maximum diameter of about 300 nm while PPRV tends to be larger, having a mean diameter of about 400-500 nm [58, 70]. The viral particle consists of an outer lipid envelop and an inner ribonucleoprotein (RNP) core. The RNP core comprises of the viral genome encapsidated by nucleocapsid protein. The RNP complex, but not the naked RNA genome, acts as a template for both transcription and replication [71]. Typically, morbillivirus genome is made up of about 16,000 nucleotides, the genome of PPRV being 15,948 bases in size, and is organized into six contiguous transcriptional units encoding six structural and two non-structural viral proteins [72]. The structural proteins are nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin (H), and an RNA-dependent RNA polymerase (RdRp) also called large (L) protein. While non-structural proteins are (C) and (V) which are transcribed by the P gene using alternative expression strategies and are found in cytoplasm of infected cells. The internal proteins (N, P and L) make up ribonucloprotein and play a role in replication and protection of viral genome while the external proteins (M, H and F) traverse the host cell derived lipid envelop and interact with host cells to transmit viral genome in to the cell [69].

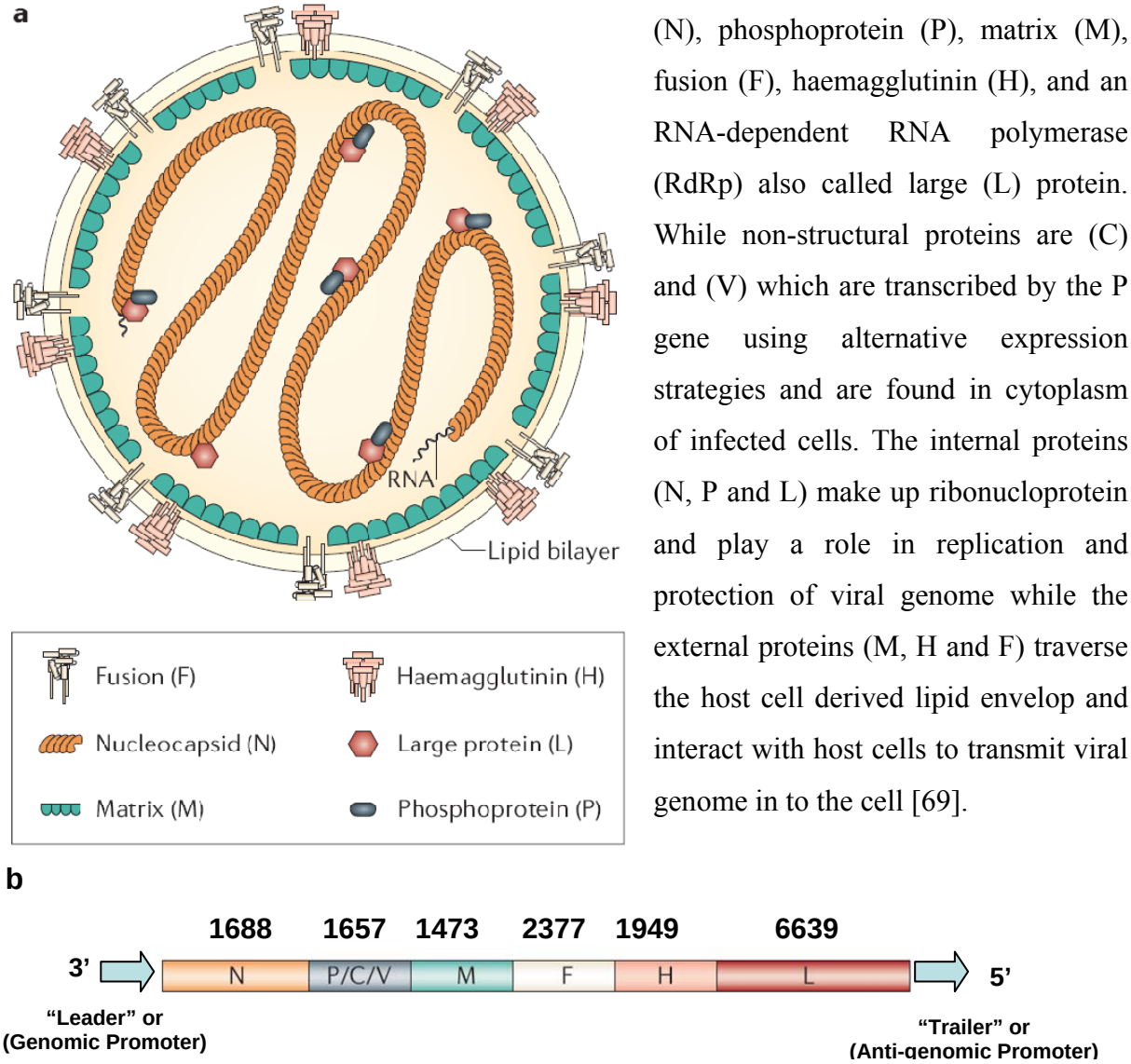


Figure 5: (a) Morbillivirus structure and (b) genome organization (Modified, according to Moss et al., 2006 and Bailey et al., 2005 [1, 72]).

The viral genome has on its 3' and 5' ends two untranslated regions (UTRs), called "leader" or genomic promoter (GP) and "trailer" or anti-genomic promoter (AGP), which control viral transcription and replication. The GP is required for both transcription of virus mRNAs and transcription of a full-length positive sense virus genome, which is a replicative intermediate, while the AGP is responsible for the production of full length negative sense genomes. For PPRV, the leader region comprises a total of 107 nucleotides with 54 nucleotides being on the 3'-UTR region of the genome followed by a stretch of 53 nucleotides at the 3' UTR of N gene [69, 72]. Whereas in case of PPRV the trailer is made of up 109 nucleotides, with 69 nucleotides at the end of the L gene followed by 40 nucleotides at the 5'UTR region. [69, 72]. Between the 3' and 5' ends lay the six transcription units which transcribe 8 viral proteins in the sequences of 3'–N-P/C/V-M-F-H-L-5'. Each of the transcription unit starts by a conserved sequence (UCCU/C) and ends with a polyadenylation signal and poly uracyl of 6 nucleotides [69]. The transcriptional units are separated by a conserved inter-genic trinucleotide 3'-GAA sequence between all viral genes except for the H and L genes where it can be GCA (MV, RPV and PPRV cote d'Ivoire/89 strain), or GAA (PPRV Turkey/2000 and the vaccine strains) and also between the L and the trailer sequence where it may be replaced by GAU (PPRV) or GUU (CDV, PDV and DMV) [69, 72]. These tri-nucleotide sequences are called mRNA pause signals for transcription as these sequences themselves are not copied.

1.3.1 Viral Proteins

1.3.1.1 Nucleoprotein (N)

N protein of morbilliviruses is a major viral protein both in the virion and in the infected cell [73]. It is highly antigenic, generating a humoral immune response [74]. Therefore many of the antibody based diagnostic tests rely upon its antigenic characteristics. Morbillivirus N proteins consist of 525 amino acid residues in RPV, MV and PPRV, but only 523 amino acids in CDV, DMV, and PMV and have a molecular weights ranging from 60 to 68 kDa [69]. The N gene of PPRV comprises 1,685 nucleotides, with 1,576 nucleotides coding for N protein composed of 525 amino acids and a molecular weight of about 60 kDa [60, 75]. The N gene is the most conserved among the *Morbilliviruses* genus [76] and the N protein is also the most conserved among viral proteins [72]. It is a structural protein which encapsidates the viral genome into an RNAse-resistant nucleocapsid (the template for RNA synthesis) and together with P protein forms the RNP, which constitutes the minimum essential for transcription and replication of viral genome in the cell cytoplasm [77].

1.3.1.2 Phosphoprotein (P)

It is one of the three components of the RNP and plays an important role in transcription and viral replication. The morbilliviral P protein is made of 506 to 509 amino acids, and has a molecular weight ranging from 72–86 kDa [60]. P protein of PPRV is the longest (509 amino acids) while the DMV counterpart is the shortest in DMV (506 amino acids) and MV, RPV, CDV and PDV have 507 amino acids [69]. It is a multifunctional protein which binds both the N and L proteins and acts also as a chaperon keeping the N in a soluble form to enable encapsidation and binding with the newly synthesized genomic RNA [52]. It is also an essential co-factor for L protein in its RdRp function [78]. P is one of the least conserved morbillivirus proteins [79].

1.3.1.3 V & C Proteins

In addition to producing the P protein, the P gene encodes the V and C proteins, via an RNA editing process and an alternative initiation of translational within a different reading frame, respectively [80, 81]. The C protein plays a role in the transcription, while the V protein plays its part in replication of viral genome. Moreover, the two proteins have a role in morbilliviral virulence as they interfere with the innate immunity by blocking interferon response by the host cells [78, 82].

1.3.1.4 Matrix (M) protein

It is a small protein with 335 amino acids and is the most conserved within the group. The morbillivirus M protein plays an important role in assembly of viral particles subsequent to transcription. [83]. It favors viral particle formation by retaining the viral RNP complex at the plasma membrane through interaction with the N protein, negatively down regulating transcription, and membrane fusion and thus promoting virus budding [84]. It associates with the inner surface of the plasma membrane [85] as well as the cytoplasmic tails of the H and F glycoproteins [85-87] on the one hand and with the RNP complexes in the cytoplasm on the other hand, to bring theses virus components together at the cell surface to make new viral particles [88].

1.3.1.5 Fusion (F) Protein

F is one of the two viral surface glycoproteins, made of 546 amino acids, which is embedded in the envelope and protrudes as spikes. It mediates fusion of the viral and cellular membranes at the cell surface and thus helps liberating nucleocapsid into the cell cytoplasm. However, before fusion can take place, morbilliviruses, except PPRV, must first attach to the host cell

receptor through its H protein, which also acts as a fusion promoter [89]. While the F of PPRV is capable of inducing fusion independent of H protein [89]. The translated F0 protein is in an inactive form which is cleaved by host cell proteases into the F1 and F2 subunits. The F1 subunit is hydrophobic and has the fusogenic properties [78]. While the virus replicates, expression of the synthesized F protein on the host cell surface results in fusion with the neighboring cells to form syncytia, which are the characteristic cytopathic effect of morbilliviruses [77]. Cell to cell fusion plays an important role in the propagation and pathogenicity of the virus. While propagating both by virus release and reinfection, the F protein on the host cell surface serves as a surface protein for the newly synthesized virus particles when they obtain envelops upon budding [77].

1.3.1.6 Haemeagglutinin (H) Protein

It is a transmembrane glycoprotein which protrudes from viral envelope and enables attachment of virus to the membrane receptors of the host cells. It mediates the interaction of the virus with the cellular receptors. In association with the fusion (F) protein, the H protein forms the envelope protein complex that is used for both virus attachment and membrane fusion. The H protein consists of 604 amino acids for DMV, 617 amino acids for MV and RPV and 609 amino acids for PPRV and RPV [69]. The H protein of PPRV and RPV is distinct in the morbillivirus group, as it possesses both the haemagglutinating and neuraminidase activities [90, 91]. Being a surface protein, the H of morbilliviruses is highly antigenic and the principal target of neutralizing antibodies. [92]. Therefore, it has to evolve to escape host immune responses and thus maintain the virus in the susceptible population. Antibodies to H protein are the second most abundant after the N protein [74]. However, N is an internal protein thus antibodies against it are not neutralizing [77]. The fact that H gene is not highly conserved, probably also reflects the role of H in binding to host cell receptors and thus playing a part in host-switching [52]. In the morbillivirus group, CDV has a comparatively wider host range and new viral variants are being found out [93]. The H gene of CDV has been found to have a much higher mean substitution rate (11.350 x 10^4 subs/site/year) as compared to measles and mumps (6,585 x 10^4 and 9,168 X 10^4 subs/site/year, respectively) [29]. It has been suggested that evolution at residue 530 and/or 549 in the SLAM-binding region of the H protein of CDV is associated with disease emergence in novel host species [51, 52].

1.3.1.7 Large (L) protein

It is the largest of the morbillivirus proteins having 5,183 amino acids and a molecular weight of about 200 kDa. It is also the least abundant viral protein as it is the last to be transcribed. Despite having a large size, the L protein is highly conserved among morbilliviruses. It functions as RdRp in association with P protein. It performs a major role in genomic RNA transcription and replication as well as the nucleotide polymerization, capping, methylation and polyadenylation of viral mRNAs [78, 94].

1.3.2 Viral mRNA synthesis and replication of genome

Upon encountering the susceptible host cell, the viral H protein interacts with the SLAM receptor molecule on the cell surface for attachment, while the F protein inserts its fusogenic domain into target cell membrane, thus initiating the membrane fusion process which is needed for entry of viral core complex into the host cell [95]. Thus the viral ribonucleoprotein core enters cell cytoplasm where it has to replicate. However because of being negative sense viruses, the genomic RNA of morbilliviruses can not be directly translated for production of viral proteins. Therefore, it is first transcribed into messenger RNAs (mRNAs) with the help of viral RdRp, which are then translated by cellular machinery to produce viral proteins. However, it is still unclear as to how RdRp accesses the template RNA which is strongly associated with N protein [69]. The viral transcription is initiated by RdRp after attachment to the GP of the viral genome. The viral genes are transcribed sequentially in a "progressive start-stop mechanism" in which, upon encountering a stop codon of a gene and a subsequent intergenic tri-nucleotide, which acts as a pause signal, the synthesized copy of RNA detaches and RdRp then moves on to the next gene. Thus are transcribed the monocistronic mRNA molecules, however occasionally the polymerase does not respect one or two inter-genic pauses, and this aberrant transcription results in synthesis of bi or tricistronic mRNAs. This progressive "stop-start" mode of transcription by RdRp also produces a gradient in the quantity mRNA produced from different morbillivirus genes. This happens because if RdRp becomes detached from RNP template during the transcription, which is frequent at gene junctions, it has to return again to the GP to reinitiate transcription. Owing to this morbillivirus genomic organization, more a gene is away (far) from the viral promoter, less it is transcribed [69]. The transcriptional gradient in protein production corresponds to the different quantities of various viral proteins needed for synthesis viral particles. Thus N protein is the most abundant while L protein is the least produced of the viral proteins (Fig.5).

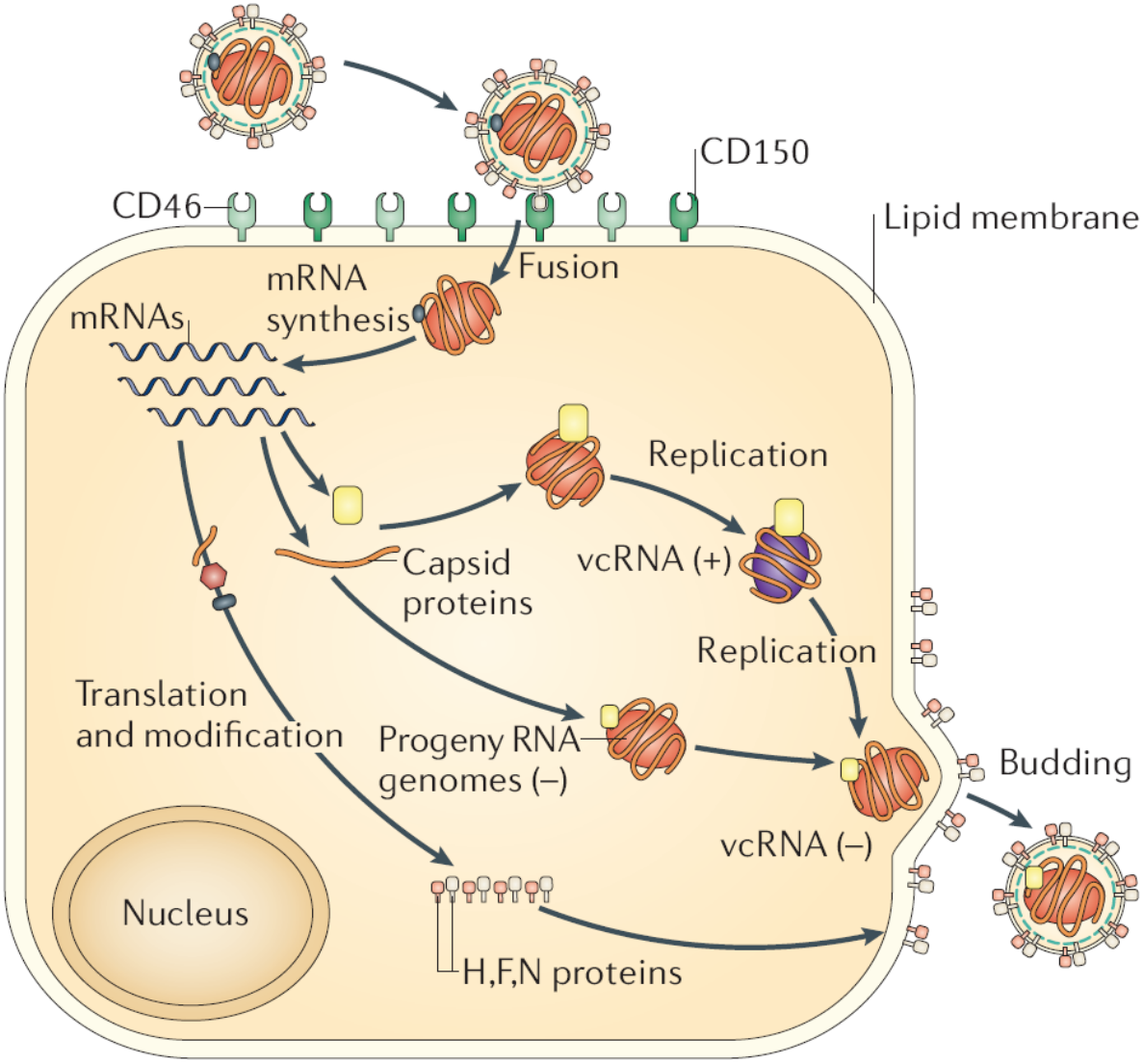


Figure 6 : Measles virus replication cycle. (according to Moss et al., 2006) [1]

Once sufficient viral proteins have been generated, gene junctions are ignored by the RdRp, mRNA transcription is stopped and assembly of a full-length, positive sense antigenome is started. The precise mechanism responsible for switching from a transcriptive to the replicative mode is not fully established. The antigenome, like genomic RNA, is also encapsidated and it serves as a matrix for synthesis of new copies of negative sense genomic RNA.

1.3.3 Replication of viral genome, assembly and release of the viral particles

The virus assembly involves association of free N subunits with newly synthesized viral genomic RNA. Next is formed the association between P-L protein forming a complex that binds with N-RNA, resulting in formation of RNP complex [77]. The H and F proteins are transported to the surface of cell membrane for incorporation on the outside of the envelope for the newly budding virions. While the M protein plays the role of a bridge, by associating on the one hand with the surface of plasma membrane, and cytoplasmic tails of the H and F glycoproteins, and on the other hand it also interacts with RNP complex [96]. Finally the interaction between viral H and F proteins on the cell membrane and RNP causes the membrane to envelope the particle and a new virion is liberated into external environment by budding. This process of budding does not kill the host cell thus allowing for (re-infection and) formation of more viral particles.

1.3.4 Host immune response

The specific antibodies may be detectable within a week of infection [97, 98] and the immune response is directed against all viral proteins. The most rapid and abundant antibodies produced by the host during an outbreak are directed against the N protein, most likely because of the location of the N gene at the 3′-end of the genome, the start–stop mechanism of the RNA-dependent RNA polymerase [99] and because the N protein is the major structural viral protein [73]. Antibodies to the morbillivirus N protein account for most of the complement-fixing activity [99], while virus neutralizing (VN) antibodies are mostly directed against the H glycoprotein and, to a lesser extent, against the F protein [99, 100]. Moreover, maternal antibodies are also transmitted to the neonates via colostrum and can protect newborns against RPV and PPRV for up to six months [101, 102]. Cell mediated immunity also plays a role against morbilliviral infections and the studies in CDV and MV show a role of cytotoxic T cells in convalescence, an immunity obtained by activation of CD4+ and CD8+ lymphocytes. A vaccination against RPV, by a Plowright vaccine strain (RBOK) of RPV [37], was found to produce a strong CD4 T cell response [103].

Although morbillivirus infections generate strong immune responses with resulting long term immunity, paradoxically, during temporary phase post-infection they also produce an immunosuppression with the emergence of secondary infections [104, 105]. PPRV infection in small ruminants also produces an impairment of cellular and humoral immune functions, resulting in frequent opportunistic infections [106, 107]. The leucopenia and immunosuppression result from a combination of factors like cell death caused by infection of

lymphocytes and apoptosis, and the cell cycle arrest, caused by exposure to the viral haemagglutinin and fusion surface glycoproteins as well as viral nucleoprotein, expressed from the damaged cells [106-108]. The immune suppression is not only observed in case of natural infections but also after vaccination with attenuated morbilliviruses [103, 109, 110].

2. Peste de Petites Ruminants : The disease, its diagnosis and control

The disease has been differently named as Kata, goat plague, pseudo-rinderpest, pneumenteritis complex and stomatitis-pneumoenteritis syndrome. However its French name has been finally adopted as Peste des Petits Ruminants (PPR). Peste des petits ruminants (literally meaning plague of small ruminants) is an acute viral disease of small ruminants clinically characterized by fever, occulo-nasal discharges, stomatitis, diarrhea and pneumonia with foul offensive breath [99]. Clinical signs are very similar to those of rinderpest.

2.1 Host range

PPR is primarily a disease of small ruminants i.e., sheep (*Ovis aries*) and goats (*Capra hircus*). Goats are more susceptible to the disease which causes heavy losses, while it can be occasionally severe in sheep [84]. However, susceptibility varies in different breeds. Apart from domestic small ruminants, PPR has also been confirmed in captive wild ungulates comprising gemsbok (*Oryx gazelle)*, Dorcas gazelle (*Gazella dorcas)*, Nubian ibex (*Capra ibex nubiana)*, Laristan sheep (*Ovis orientalis laristanica)*, and Nilgai (*Boselaphus tragocemalus)* [2]. Experimentally, the American white-tailed deer (*Odocoileus virginianus)* is fully susceptible [111]. The only case of wild animal being seropositive with PPRV is that of African grey duikers (*Sylvicapra grimmia*) which were found to be seropositive in Nigeria [112]. PPR is primarily a disease of small ruminants, but infected cattle, buffalos, and pigs that develop unapparent infections and seroconversion are considered as epidemiological dead-ends. However, PPRV virus was isolated from pathological samples in buffaloes in India [5]. Camels in Ethiopia and in Sudan showed clinical signs of the disease and proved to be infected by PPRV [3, 4].

2.2 Transmission

The infected animal can excrete PPRV in discharges about 24-48 hours before onset of clinical signs [97, 113]. The secretions from eyes, nose and mouth as well as feces contain large quantities of the virus [113]. In an experimental infection of goats with PPRV, virus could be detected in ocular secretions on day 2 and in nasal and oral secretions on day 3 post-infection through RT-PCR [91]. Since the virus cannot survive long outside the host body and

the fact that upper respiratory tract is the entry point of the virus in to the host, close contact of infected animal with the susceptible animals is an essential requirement for transmission as described for RPV [114, 115]. The transmission occurs either through inhalation aerosol dispersed virus or by ingestion of infected material [116-118]. There is no carrier state.

2.3 Pathogenesis

Like other viruses in the *Morbillivirus* genus, PPRV is also lymphotropic and epitheliotropic [61]. The viral tropism is also manifested by the clinical signs like occulo-nasal discharges, diarrhea, oral erosive lesions, marked immuno-suppression, and leucopenia [119]; while viral antigen can be found in lymphoid organs and epithelial tissues of the affected animals [106]. The SLAM or CD150 is the primary receptor for PPRV [120], which is found on activated T cells, B cells, thymocytes, macrophages and dendritic cells [121, 122]. The viral H protein interacts with the SLAM receptor molecule on the cell surface while the F protein inserts its fusogenic domain into target cell membrane, thus initiating the membrane fusion process which is needed for entry of viral core complex into the host cell [95]. For PPRV infection, respiratory route is the portal of entry as is the case of measles. It has been found that in primate models as well as in mice expressing the MV receptor human SLAM the sub-epithelial dendritic cells are initially infected by MV in the air ways [123]. However DCs in respiratory epithelium express very low level of CD150 receptors [124]. These DCs express C-type lectin called DC-SIGN which has been postulated to have a role in MV capture and transmission to the lymphocytes in the regional lymph nodes by *in trans*–infection (independent of *de novo* synthesis of virus in infected DCs) and also *in cis* infection (by the newly synthesized of viruses in infected DCs) in combination with CD150 receptor [124, 125]. Similar role of DC-SIGN receptors have also been found in pathogenesis of several other viruses like human immunodeficiency virus (HIV) [126] and herpes simplex virus (HSV) [127]. Infected DCs also carry partly processed viral antigen to the lymphocytes in the lymph glands [42, 128]. The immunological synapse formation, with lymphocytes in the lymph glands, enhances viral transmission due to prolonged interactions during antigen presentation. Furthermore antigen presentation initially activates them and activated lymphocytes expressing greater CD150 are later infected [124]. While primary targets of morbilliviruses are the CD150 (SLAM) positive cells, for release from the host in the later stages of infection, the virus uses an additional unknown receptor(s) on SLAM-negative epithelial and endothelial cells [129, 130]. Interestingly, the epithelial cells are infected basolaterally only upon contact with underlying already infected leukocytes [128]. Since, the

epithelial cells can only be infected by MV via basolateral surface; role of respiratory epithelium in initiation of infection is not an essential pre-requisite [95].

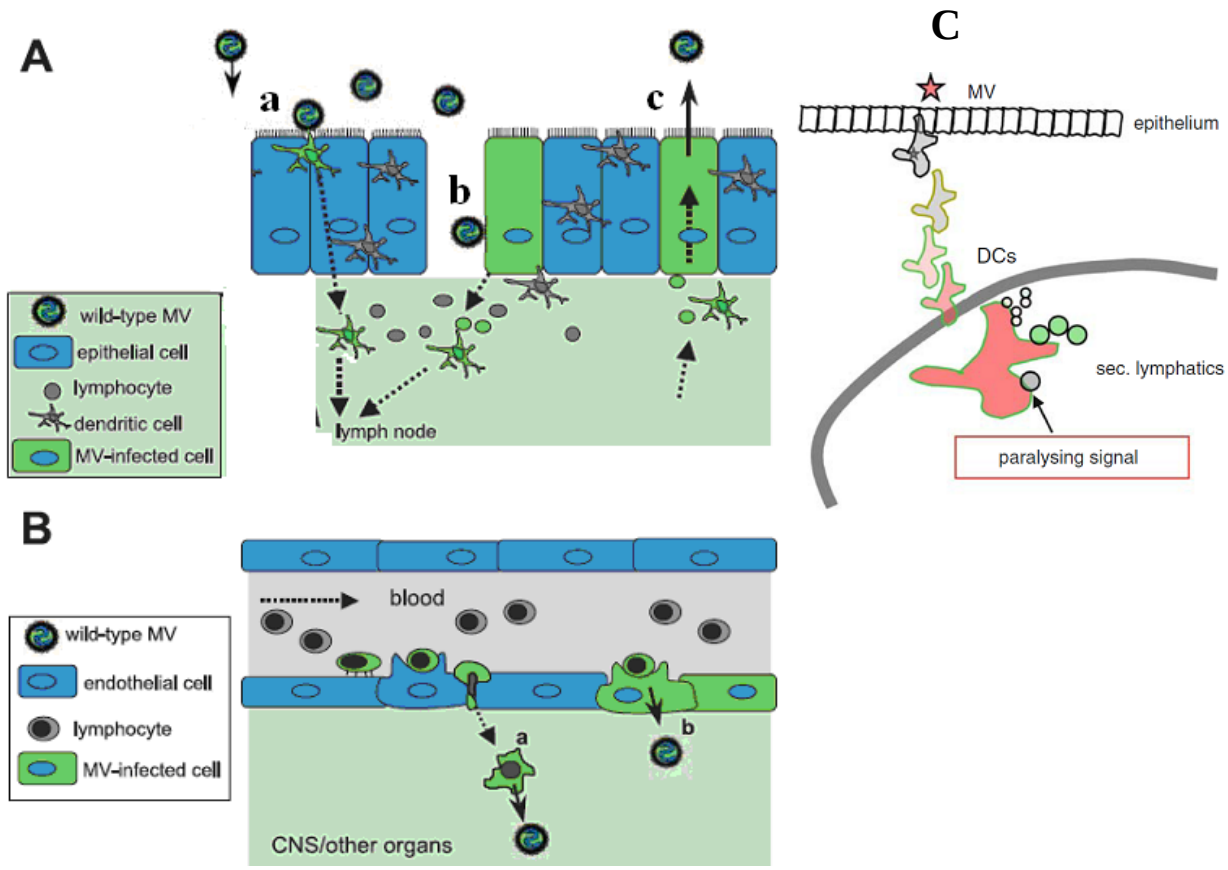


Figure 7: Possible strategy used by measles virus to cross epithelium of lung (A) and endothelial barriers (B). Antigen presentation and transmission of measles virus by the infected DCs to lymphocytes in the lymph glands (C) [42, 95] Modified.

Moreover, after intra nasal inoculation of rhesus monkeys with a recombinant MV selectively unable to recognize human SLAM (SLAM-blind), it induced neither clinical signs nor significant viremia, proving that efficient SLAM recognition is necessary for viral virulence and pathogenesis [131]. Additionally, in experimental infection of ferrets with canine distemper virus expressing GFP, it was found that CDV replication in epithelial cells was not detectable initially, but was found substantial before death of the host [132]. In the same experiment, 40% of T and 60% of B lymphocytes were infected [132].

Even after recovery from PPRV infection, there is a long lasting and important inhibition and impairment of cellular and humoral immune functions characterized by immuno-suppression, lymphocyte loss and leucopenia rendering animals highly susceptible to opportunistic infections [106, 107]. The marked immune-suppression and leucopenia may be due to a combination of cell death, caused by infection of lymphocytes and apoptosis, and the cell

cycle arrest, caused by exposure to the viral haemagglutinin and fusion surface glycoproteins as well as viral nucleoprotein, expressed from the damaged cells [106, 107].

2.4 Gross and microscopic pathology

Animals dying from the peracute form of PPR do not show any gross pathological findings except for congestion of oral mucosa and ileo-caecal valve and occasional oral erosions. However, major gross lesions are mostly found after death with an acute form of the disease. On digestive system, erosive and necrotic lesions are found on the lips, tongue, gingiva, hard palate, and oral mucosa. Occasionally, lesions may be even found on pharynx and erosions on the ruminal pillars and abomasum. Congestion of intestinal mucosa, colon, rectum with some hemorrhages and thickening of mucosa giving a zebra-stripe appearance, are also observed. The gut-associated lymphoid tissues are necrotic and mesenteric lymph nodes and spleen may be swollen. Respiratory mucosa is congested with erosions in nares and trachea. There is fibrinous or suppurative pneumonia in cardiac and apical lobes of lungs. Pulmonary lymph nodes are found swollen and oedematous. In females, vulvo-vaginitis may be seen. Atypical renal lesions have also been reported [133].

The histopathological findings in gastrointestinal tract include degeneration and necrosis of labial mucosa, mucosal and submucosal congestion, degeneration and necrosis of intestinal epithelium and lymphoid cell depletion in Peyer's patches. Mucosal alterations are surrounded by a mild inflammatory infiltration of macrophages and lymphocytes at the borders of the lesions [133]. There is lymphocytolysis and occasional syncytia formation in lymphoid tissues. The viral antigen can be found in conjunctival, tracheal, bronchial, bronchiolar and ileal epithelial cells. It is also found in pneumocytes, macrophages, and lymphoid and reticular cells in the lymphoid organs [106]. The viral antigen is mostly found on the apical portions of the proliferating surface epithelium on the luminal side [134].

2.5 Clinical signs

The length and severity of disease may vary according to species or breed involved, along with age and immune status of the animal. The PPR clinical description [54, 135, 136] can be divided in four forms, i.e., peracute, acute, subacute, and subclinical.

The **peracute form** is seen when PPR occurs in young and naive populations of sheep and goats. It starts after an incubation period of about 1 to 3 days and is clinically characterized by high fever (40-42°C), dullness, anorexia, and constipation. There is congestion of oral and

ocular mucosa initially, however, after two to three days of pyrexia, occulo-nasal discharges are observed and oral mucus membranes are occasionally eroded. The affected animals develop profuse watery diarrhea and die within 4 to 6 days after onset of hyperthermia.

The **acute form** is the most frequent. The first symptoms arrive after 3-4 days of incubation and include sudden hyperthermia and mucosal congestion. Diarrhea starts 2-3 days after the onset of fever. Animals become depressed, anorexic, dehydrated and have serous occulo-nasal discharges (which may later become muco-purulent). The epithelial lesions are mostly found on lips, gums, dental plate, tongue, and also on labial surface of vulva in female animals. Pregnant animals may abort. There are signs of bronchopneumonia with occasional productive cough. The muco-purulent discharges in later stage may be followed by crusting of nares, causing sneezing and coughing. Finally, there is hypothermia, prostration, and death may occur 10-12 days after onset of fever, some animals may survive for three weeks. Mortality rate may reach 70-80%.

The **subacute form** of the disease is seen after about one week of incubation period and is clinically characterized by a low grade fever and either less severity or absence of other clinical signs. Many animals will recover after about 10-14 days of illness.

In the **subclinical infection**, no clinical signs appear and can only be diagnosed serologically. The clinical picture may change during course of infection by complication due to secondary bacterial infections of oral, intestinal and respiratory tracts. Furthermore, PPR is characterized by immuno-depression, and this may activate latent intestinal or blood parasites [133].

(a) (b)

Figure 8: (a) Oral lesions and (b) nasal discharge caused by PPRV infection in goats (Source: Abdallah Traore, LNRV, Bamako, Mali)

2.6 Diagnosis

PPR can be diagnosed on the basis of clinical signs, as well as laboratory tests. The clinical diagnosis is based upon major clinical signs of PPR like which include fever, oral erosive lesions, dyspnoea, and diarrhoea. However, definitive diagnosis is possible only with laboratory tests.

2.6.1 Differential diagnosis

Certain clinical signs manifested in PPR in small ruminants can also be found in some other infectious diseases of sheep and goats.

- **Rinderpest**: PPR has long been confused with rinderpest however unlike PPR there no respiratory problems associated with RP. Moreover, RP is almost eradicated now.
- **Pasteurellosis**: It does not have involvement of digestive system, however may exist as a secondary infection alongside PPR.
- **Contagious ecthyma**: similar to subacute form of PPR, however the lesions are not erosive but are in the form of papules or pustules which sometimes be found on mouth and nose.
- **Foot and Mouth Disease**: Oral lesions are present but there is absence of respiratory problems and diarrhoea
- **Bluetongue**: affects mainly the sheep but rarely the goats and is characterized by signs of congestion of oral and nasal congestion and edema of lips and tongue there is oedema of the face which are not found in PPR.
- **Contagious Caprine Pleuropneumonia**: is not characterized by diarrhea or oral erosive lesions.

In case of suspicion, the confirmatory diagnosis can only be based on laboratory tests. The samples can be collected either from the living sick or freshly died animals. Blood samples with and without anticoagulant should be taken for obtaining infected peripheral blood mononuclear cells (PBMC) and serology, respectively. Swabs or nasal and ocular discharges should be taken. Tissue samples should also be taken from thymus, mesenteric and bronchial lymph nodes, lung and intestines. The samples must be rapidly transported at 4°C to laboratory for diagnosis. For rapid sample collection, blood or nasal and ocular discharges can also be sampled on filter papers, dried and stored over long periods (up to 9 months) at ambient temperatures [137].

The laboratory tests for diagnosis of PPR are based upon isolation of virus, detection of viral genome, and detection of viral antigen or antibodies. Virus isolation is the classical technique for diagnosis and still serves as a gold standard. In past, primary kidney or lung cells of goat or Vero cell (kidney cells of African green monkey) cultures were used, which required numerous blind passages and the isolation was often not efficient [138]. However, since discovery of the fact that SLAM is the common receptor of morbilliviruses [139], Vero.DogSLAMtag (expressing canine SLAM receptor) [140] and CV1 cell line (expressing sheep SLAM receptor) [141] have been developed in recent years, which have improved PPRV isolation *in vitro*. Cytopathic effects (CPE) induced by the virus in these cells can be observed much earlier, in fact within two days after infection instead of 2-3 weeks for the normal vero cells [141]. However, for virus isolation good quality and well conserved samples are still required.

Various diagnostic tests are available which are based upon detection of viral antigens like agar gel immunodiffusion (AGID), and immunocapture ELISA (Enzyme-linked Immunosorbent Assay [142]. While for viral antibody detection tests like virus neutralization test (VN), indirect ELISA and competitive ELISA are used [143]. Among these test, AGID is incapable of discriminating between PPR and RP but immunocapture ELISA based monoclonal antibodies [144, 145] against PPRV or RPV can efficiently discriminate between the two viruses and have proven to be rapid, sensitive and specific tests (115). Numerous diagnostic tests which use nucleic acid based detection of the pathogen which includes use of radioactive or non-radioactive DNA probes, conventional reverse transcription-PCR (RT-PCR), quantitative RT-PCR (QRT-PCR) [142, 146]. PCR based diagnostic tests are highly specific and, using specific primers, can not only discriminate between PPRV and RPV, but are also capable of discriminating between lineages of PPRV.

2.7 Prophylaxis and treatment

To prevent the spread of PPR to new areas, measures like controlling the movement of animals from the areas endemic for PPRV is of primary importance. However, in case of an outbreak of the disease in a new area, the most effective solution would be to kill the infected animals and destroy the cadavers [36]. However, PPR is endemic in developing countries therefore stamping-out policy is seldom accepted due to high costs. The only viable prophylactic measure is vaccination of the susceptible animals around the affected area. Effective vaccines are available against PPRV since long time. Rinderpest vaccine, being cross protective against PPRV, was used initially but it can no longer be used due to Global

Rinderpest Eradication Program. Indeed, rinderpest vaccination was banned in the process of eradication to allow effective epidemio-surveillance of the circulation of any residual virus and avoid confusion between infected and vaccinated animals using the available antibody detection tests. An effective homologous vaccine is available since 1989 [147], which provides immunity against the disease during the economic life of small ruminants.

There is no effective treatment against PPR, once animals are infected. The sick animals can only be provided supportive and symptomatic therapy. Antibiotics are also given to prevent secondary bacterial infections. Not much research has been done for discovery of antiviral agents effective against PPRV. As for as small molecular drugs are concerned, there is only one report of *in vitro* testing of a synthetic compound 4,4′-(arylmethylene)bis(1*H*-pyrazol-5-ols) for its ability to inhibit CPE produced by PPRV and this compound was found to be more potent than the standard drug ribavirin [148]. The *in vivo* use of classical small molecular antiviral drugs has serious limitations due to toxic side effects and emergence of resistant strains [149]. The antivirals based RNA interference (RNAi) are a promising approach as they are highly specific end effective in their action. However, even after more than a decade after their discovery, their development as a therapeutic tool is hindered by problems of *in vivo* delivery. In fact, problems are somewhat similar as the gene therapy has been facing since 1980s.

3. Interfering RNAs as antivirals and their delivery vectors

3.1 RNAi

RNAi is a highly specific, evolutionarily conserved, post transcriptional gene silencing mechanism used by cells for gene regulation, protection of genome against transposable elements and the attack of RNA viruses. While RNAi plays an important innate antiviral defense mechanism in plants and insects [150, 151], whether RNAi as antiviral immune mechanism really exists in mammals, remains unclear [152, 153]. However, the enzymatic machinery for RNAi is used by the other endogenous non-coding small RNAs like microRNAs (miRNAs), for regulation of gene expression [154, 155]. It is estimated that RNAi mediated gene regulation controls the expression of about 30% of mammalian genes, many of which are involved in functions like cell fate, proliferation and death [156].

3.1.1 Discovery

The phenomenon of RNAi was first observed by Napoli *et al* in 1990, when they were trying to over express an enzyme responsible for plant coloration by introduction of the exogenous gene in petunias [157]. Surprisingly, the introduced gene resulted in a blockage of pigment synthesis. The phenomenon of gene suppression, however, was not understood at that time. It was Andrew Fire and Craig Mello who finally established the mechanism in 1998, by demonstrating that injection of short stretches of 23mer to 25mer nucleotide sequence of double stranded RNA (dsRNA) into the nematode *Caenorhabditis elegans* resulted in silencing of endogenous genes [158]. Fire and Mello were awarded the Nobel Prize for Medicine or Physiology in 2006 for their discovery. This mode of gene silencing was different from the already established anti-sense mode of gene silencing in that, although both are based upon sequence complementarity to messenger RNA (mRNA). RNAi has a catalytic component that makes it possible for a single small interfering RNA (siRNA) molecule to bind with and destroy thousands of copies of mRNA molecules and thus is 1000 times more effective than antisense oligonucleotides (ODNs) in silencing target gene [159-161]. Subsequently, it was reported that RNAi could take place in numerous other organisms including invertebrates, and vertebrates [125, 126]. Elbashir *et al* in 2001, showed that transfection of synthetic siRNAs could silence genes in mammalian cells [127]. Since then RNAi has found widespread applications ranging from use in functional genomics and gene knock-down, to treatment of various medical conditions like macular degeneration, cancers and viral infections.

3.1.2 Mechanism

RNAi machinery in mammals is used by miRNAs for regulation of gene expression. The miRNAs are transcribed by polymerase II as long primary transcripts or pri-miRNAs [162]. The pri-miRNAs undergo a sequential maturation process in which they first bind to a microprocessor, a complex of two proteins in the nucleus which contains the DiGeorge Syndrome critical region-8 (DGCR8) protein and a nuclear RNase III called Drosha [163-165]. Next the Drosha cleaves pri-miRNAs into pre-miRNAs, which are the imperfectly paired stem-loop miRNA precursors having 60 to 80 nucleotides. The pre-miRNAs are then exported to the cytoplasm by Ran GTP-dependent Exporting-5 transporter [166, 167]. The Dicer (an RNase III family enzyme) at first, interacts with its double stranded RNA-binding protein partner (TAR RNA binding protein; TRBP) and other partners to cleave pre-miRNAs into mature double-stranded miRNAs of 19-25 bp with 2 nucleotide 3’ overhangs [168]. Then, Dicer together with R2D2 protein and its partners, couple with miRNAs duplex to form

RISC loading complex (RLC), which helps in the loading of the miRNA duplex into another multiprotein complex, having Argonaute protein as its core component. This new complex is called RNA-induced silencing complex (RISC or miRNA protein complex (miRNP)) [155, 169, 170]. After attachment to RISC, the passenger strand of miRNA is lost, while antisense strand guides RISC to the target mRNA. The miRNAs pair perfectly or imperfectly with the target mRNAs and thus may either result in endonuclease Argonaute 2 mediated cleavage and destruction of guide strand-mRNA duplex or blockage ribosome movement halting mRNA translation, respectively [171].

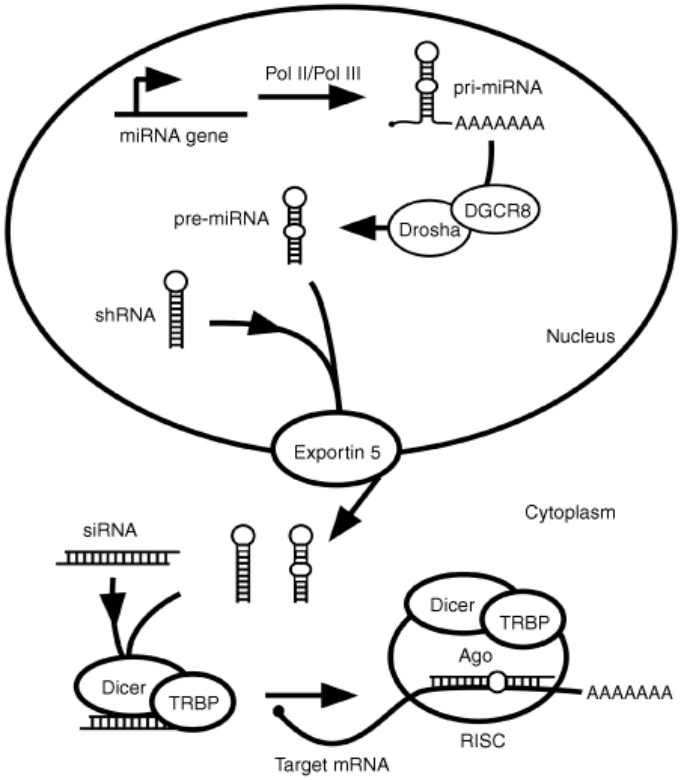


Figure 9: Mechanism of RNA interference (Schematic representation - according to Keck, 2008) [172]

The degree of miRNA-mRNA complementarity has been considered to be the key determinant of the regulatory mechanism. Thus, in case of a perfect match, mRNA is cleaved by RISC, while central mismatches may result in repression of mRNA translation [154]. The plasmid or viral vector expressed short hairpin RNAs (shRNAs) and chemically synthesized siRNAs mimic mammalian pre-miRNAs and miRNAs respectively and thus use cellular machinery for gene silencing. The only difference being that, the antisense strand of siRNA, acting as a guiding strand pairs perfectly with the target mRNA resulting in RISC-mediated cleavage of the target mRNA [173]. Viral/plasmid vector based shRNAs, transcribed from a polymerase III promoter, are produced in the nucleus and exported to the cytoplasm by exportin-5 together with GTP-bound form of its cofactor Ran, and are processed by Dicer like pre-microRNAs to produce siRNAs [167].

3.1.3 Interfering RNAs as an antiviral therapeutics

The infection by negative sense RNA viruses requires the transfer of viral genome into the host cells, transcription of the viral mRNA, and translation of the viral proteins that are needed for viral genome replication, assembly, and budding. Targeting viral mRNA with siRNA is an attractive strategy since firstly, one can prevent synthesis of critical viral proteins to disrupt viral life cycle, secondly with siRNAs, being highly specific, there is little chance of side effects, and finally, understanding of gene function is not required: only viral genome sequences are needed [174]. Soon after discovery of RNAi, its potential as effective antiviral therapy was recognized. RNAi antiviral treatment was first used by Bitko and Barik in 2001 against respiratory syncytial virus (RSV) [175]. Since then the approach has been variously exploited for antiviral purposes, not only through the use of chemically synthesized siRNAs but also by expressing the shRNAs from plasmid or viral vectors [176-178]. RNAi through these various approaches could effectively block viral replication *in vitro* but for *in vivo* use, delivery issue is still a major hurdle.

3.1.4 Difficulties in use of RNAi as an antiviral approach

The therapeutic use of RNAi *in vivo* faces other practical difficulties than those related with delivery, like off-target effects, RNAi suppression and viral escape.

3.1.4.1 Off-target effects:

Although RNAi is highly specific, in addition to the intended mRNA suppression, it also produces unintended effects on gene expression. For *in vivo* delivery, off-target effects are major concerns since the down-regulation of self genes could have serious biological consequences. They may result from either a partial sequence complimentarity of RNAi construct to non-targeted mRNA or induction of a variety of immune and toxicity related effects emanating from certain motifs or patterns in the RNAi construct itself [179].

3.1.4.1.1 Specific off-target effects dependent on siRNA sequences

Although initially thought to be highly specific, RNAi was shown by expression profiling to produce off-target effects whereby a particular siRNA will bind to other mRNAs than the one originally targeted. Partial sequence complementarity in both the passenger or guide strands of RNAi construct can produce off-target gene suppression [180]. The off-target silencing has been reported for transcripts with as low as 7 nucleotides complementarity with the guide strand [146]. siRNAs like miRNAs can also bind to sequences with partial complementarity

at the 3'-UTR rather than overall homology between the siRNA and targets [181-183]. Furthermore, both the siRNA and shRNA with a complementarity in the "seed region" can produce similar off-target expression profiles [184], however shRNA is reported to induce fewer off target effects than siRNA [185].

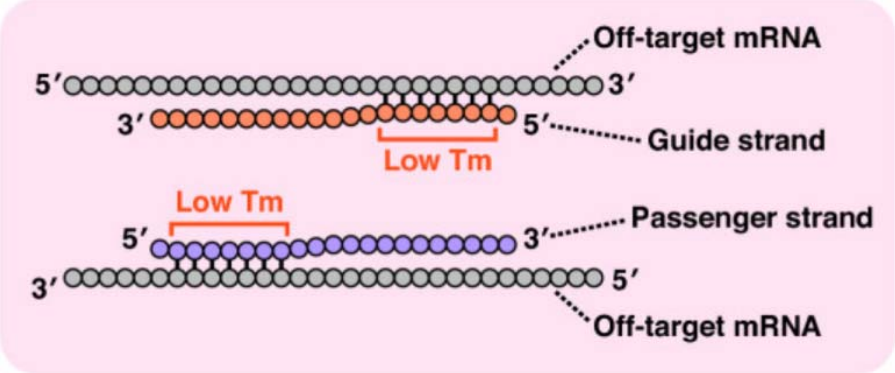


Figure 10: Seed dependant off-target effect. The capability of siRNAs to induce seed-dependent off-target effect is highly correlated to the thermodynamic stability of the duplex formed between the seed region of siRNA guide strand and its target mRNA [186, 187].

3.1.4.1.2 Non-specific off-target effects - Induction of type 1 interferon (IFN) and other immune responses

The animal immune system can discriminate between self and non-self nucleic acids. The double-stranded RNA (dsRNAs) longer than 30 bases can induce an immune response via protein kinase R (PKR), resulting in a general degradation of mRNA and inhibition of translation as well as up regulation of interferon (IFN) stimulated gene expression [188]. Moreover, even the smaller (<30 nucleotides) siRNAs, although initially considered non-immunogenic [173], have been found to induce a partial cytokine and type-1 IFN response via toll like receptors (TLRs) [189-191]. It is also reported that although RNA sensing receptors are also found in the cytoplasm, nucleic acids mediate immunoactivation mainly through TLRs 7 and 8 (activated by ssRNA), TLR9 (by unmethylated CpG motifs in bacterial plasmids) and TLR3 (via dsRNA) [192-196]. siRNA immune stimulation via TLR7 and TLR8 on endosomes can be sequence dependent and therefore is possibly avoidable [191, 193, 197]. The chemically synthesized siRNAs delivered by transfectants enter cells through endocytosis and are therefore more prone to cause immune stimulation via TLRs 7/8 found on endosomes. Conversely, shRNAs expressed intracellularly by plasmid and viral vectors, follow more closely the endogenous RNAi pathways and thus prevent interferon response [179, 198].

3.1.4.2 Saturation of endogenous RNAi pathway components

The enzymatic machinery involved in RNAi in mammalian cells is naturally used by miRNAs which play an important role in cell physiology. Since miRNA and shRNA share common enzymatic pathway for their processing, the over-expression of shRNA *in vivo* is known to produce toxicity due to over-saturation of endogenous miRNA pathway involving exportin-5 and RISC component Argonaute-2 [199]. However, in vivo toxicity can be alleviated by the selection of efficient but safe shRNA expression cassettes, and applying minimal effective vector doses [199].

3.1.4.3 Viral encoded suppressors of RNAi

Several plants and invertebrates use RNAi as an immune mechanism to counter viral infections [150, 151]. Therefore, viruses infecting these plants and insects have evolved their countermeasures by producing various suppressors of RNAi silencing (SRS) [200, 201]. Some recent studies suggest that RNAi in mammals is also involved in antiviral responses and cellular regulatory miRNAs have a function in restricting virus replication in the cells [152]. Moreover, several mammalian viruses like hepatitis C virus (HCV), influenza virus, ebola virus, human immunodeficiency virus (HIV), vaccinia virus, and adenoviruses type 2 and 5 are known to produce SRS factors that inhibit the RNAi mechanism. Many of these factors are multifunctional proteins which are known to inhibit interferon response as well [152, 153]. Since the viruses producing SRS can be efficiently inhibited by RNAi, it appears that viral-suppression activity does not pose a serious difficulty for therapeutic RNAi [202]. None of the viruses belonging to the morbillivirus genus is reported to produce RNAi suppression through SRS factors.

3.1.4.4 Viral escape

Several applications of a single siRNA can lead to the emergence of viral escape mutants due to mutations in the target sites making viral genomes resistant to RNAi [203]. Since, unlike eukaryotic DNA polymerase, RNA viruses lack proofreading activity, they tend to have a high error rate during replication which facilitates development of viral mutants [204]. After discovery of RNAi, an early study by Jacque *et al*., 2002, showed that even a single nucleotide mismatch in the siRNA target region may be sufficient to reduce the silencing effect of an siRNA on HIV-1 [205]. Interestingly, viral escape to RNAi can also occur by a point mutation outside the target sequence of siRNA, if this mutation changes local RNA folding into a structure that reduces the accessibility of the target sequence [206]. Several viruses have been reported to escape suppression by effective siRNAs, which include HIV-1,

HCV, HAV, and Poliovirus [207-210]. Studies of HIV-1 and HBV populations have shown that many resistant mutants may pre-exist before they have been exposed to inhibitors and the siRNAs may also exert selection pressure on these pre-existing resistant mutants [211, 212].

The problem of RNAi escape can be more severe in chronic diseases as these require an extended antiviral therapy but may be less crucial in treatment of acute diseases [202]. However, design and selection of siRNAs targeting conserved regions of virus and combination of siRNAs targeting multiple genes or regions in viral genome can help avoid problem of resistance to siRNA therapy [213]. In a study using a T cell line expressing three potent shRNAs against HIV-1, it was found that even after an extended culturing for more than 100 days there was no viral replication [214]. A combination of four shRNAs that target different sequences of HBV genome has entered a phase I clinical trial [215]. However, use of multiple siRNA/shRNA doses must be optimized before use as some studies have shown that it is possible to saturate the RNAi pathway with high levels of shRNA expression [199]. Another possible alternative to targeting multiple conserved viral genomic regions is to use siRNAs that recognize the mutated target sites through use of siRNAs or shRNAs that target the most likely escape variants [216]. *In vitro* studies are needed to study the frequency of emergence of PPRV escape mutants and the viral sequences that are likely to mutate in the course of therapy. Since viruses tend to mutate, an alternative means could be to down-regulate cellular factors which are required by the virus to either enter a cell or to replicate.

3.2 *In vivo* delivery of interfering RNAs

Compared to the large number of studies using siRNAs in cell culture, there have been relatively few studies *in vivo*. The progress is slower due to difficulties in delivery, especially by systemic route of administration [217]. The issue of drug delivery is not unique to RNAi based therapeutics, but here it is a major obstacle as the drug cargo (siRNA) is nuclease sensitive, has a net negative charge, and is hydrophilic, thus making difficult to keep it stable in serum and capable to cross the anionic and hydrophobic plasma membranes [218-220]. Moreover, for diseases like PPR, systemic delivery of siRNA is a necessity. In order to use RNAi as an antiviral approach, the siRNAs can either be chemically synthesized [175] or shRNA expression cassettes with Poll III promoters can be inserted into plasmid [176] or viral vectors [177, 221]. The viral vectors can efficiently use their own machinery to reach the target cells, transfer their genome into the cells and express shRNA molecules.

The naked siRNAs and plasmid vectors, however, can not readily cross the cell membrane and therefore need delivery vehicles to help them to enter the cell cytoplasm. Bitko and colleagues successfully delivered naked siRNAs intranasally in a mouse model against

respiratory syncitial virus [19]. Interestingly, they compared delivery of siRNA with and without a transfection reagent, and noted only a marginal enhancement (20%) in the knockdown of the respiratory syncytial virus (RSV) target gene when lipid agent was used. However, the mechanism by which these cells take up these siRNA molecules remains unknown [222]. The systemic delivery route, however, is more difficult to approach. The unmodified siRNA has a half-life of less than an hour in human plasma and the siRNA molecules circulating in blood, are rapidly excreted by kidneys due to their small size [223]. The vectors are therefore needed not only to improve the bio-availability of siRNAs but also to provide protection from nucleases and help in penetration of anionic plasma membranes [224, 225]

3.2.1 Physical methods of siRNA delivery

The physical methods for *in vivo* delivery of siRNA involve mainly electroporation and hydrodynamic injection. The hydrodynamic injection involves a quick injection of siRNA or plasmid DNA (pDNA) in a large volume of physiological buffer, around one-tenth the mass of the animal, within a few seconds in the tail vein of the animal and this results in a delivery mainly to the liver [226, 227]. The early attempts for systemic delivery of siRNAs *in vivo* used the hydrodynamic injection method that was already tested by Liu *et al.,* for pDNA delivery in mice [228]. McCaffrey *et al.,* in 2002, in their studies on *in vivo* siRNA delivery by hydrodynamic method, demonstrated that co-injection of luciferase expressing plasmid with siRNA targeting luciferase produced efficient silencing of luciferase expression in the mouse liver [229]. Various studies using this method could deliver naked siRNA to hepatocytes, demonstrating functional knockdown of specific genes in the livers of mice [229-231]. Most studies are using hydrodynamic method for delivery of siRNA to liver as it can be much more easily transfected by this method as compared to other organs. This procedure is not clinically viable because of potential damage to liver, as well as due to danger of volume overload side effects like right sided heart failure [232]. Moreover, it would be very difficult to scale up this method for use in large animals.

The electroporation involves administration of an electric current for short duration, which temporarily increases the permeability of cell membranes, and thus allowing for passing of nucleic acids through membranes. The electric field polarizes the membrane molecules and temporarily destabilizes the membrane integrity, which results in greater permeability for the exogenous materials [233]. This technique has been successfully used to transfer siRNAs locally [234], but it cannot be used for systemic administration. Similarly, ultrasound is also

used for nucleic acid delivery [235]. These physical methods of delivery of siRNAs either cannot be used systemically or are too dangerous for clinical use.

3.2.2 Chemical vectors for delivery

The development of chemical vectors for siRNA delivery has been influenced by the studies on intracellular DNA delivery [236]. However, there are important differences between delivery of siRNA and DNA: i) size and charge of siRNA are lower than those of DNA; ii) cytosol is the place of action for siRNA while DNA has to enter nucleus to be effective [237]. The chemical methods of siRNA delivery involve formulation of negatively charged siRNA molecules with various polycations, like cationic lipids, peptides, polymers, or inorganic nano-structured materials, either through electrostatic or ionic interactions, with the aim of enhancing pharmacokinetic behavior, nuclease resistance, cellular uptake, target specificity and safety [18]. The general principle is based on complex formation between anionic siRNA molecules and cationic polymers resulting in complexes with net positive charge for facilitating interaction with anionic cell membranes. Moreover, the physico-chemical properties of the complexes like size, morphology, surface charge and stability, are the major determinants of transfection efficiency which in turn are determined by structure of polycation, the polycation/nucleic acid stochiometry (+/- charge ratio), pH and order of mixing of nucleic acids and the vectors [238]. Though most delivery vectors are cationic, the net surface charge of the vector-siRNA complex, has to be near neutral to avoid toxicity, excessive uptake by reticulo-endothelial system (RES) and aggregation by serum proteins [18, 239]. In order to deliver siRNA cargo into target cell cytoplasm, the formulation has to surpass various physiological barriers (Fig. 11) by shielding siRNA from serum nucleases, avoiding aggregation by anionic serum proteins, escaping from RES, penetrating endothelium, getting internalized through endocytosis, and finally being able to deliver siRNA from the endosomes to the cytoplasm before degradation by lysosomes [18, 239]. The *in vivo* use of siRNA by chemical delivery systems is limited by siRNA packaging efficiency, colloidal stability of the complex, internalization, and endosomal escape [240].

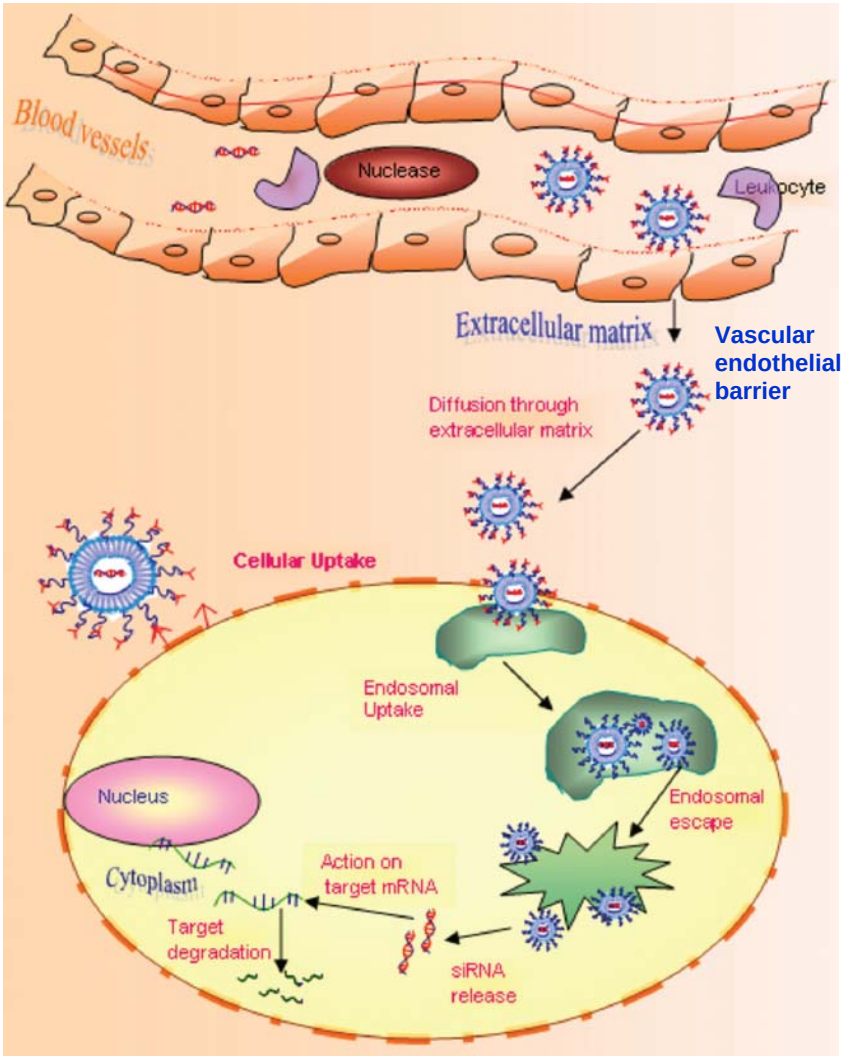


Figure 11: Main physiological barriers to *in vivo* delivery of siRNA (Schematic representation according to Misra *et al*., 2010) [18]

The chemical vectors used for siRNA delivery can be broadly divided into three major groups: lipids, cationic polymers, and peptides.

3.2.2.1 Lipids as nucleic acid delivery vectors

Bangham *et al*., (1965), while studying cell membranes, observed that phospholipids which are a major component of cell membranes, tend to form spontaneous spherical structures called liposomes upon contact with water [241]. Latter, Felgner *et al.,* (1987), first brought demonstration of effective use of liposomes for in vitro transfection of DNA [242]. These liposomes were based on a cationic lipid called DOTMA (N-[1-(2,3,-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) [242]. Since then, many natural as well as synthetic cationic lipids have been developed and used for delivery of nucleic acids including DNA, antisense oligonucleotides and siRNAs. The preparation of these new cationic lipids involved either modification of the nature of cationic lipids [243], fatty acid side chains [244], or

formulations with some additional lipids [245]. Despite initial difficulties found in *in vivo* transfection, the death of a human patient participating in gene therapy trials based on an adenoviral vector in 1999 [246] and the resulting temporary ban on use of viral vectors for gene therapy by US Food and Drug Administration [247], lead to a renewed interest in chemical vectors.

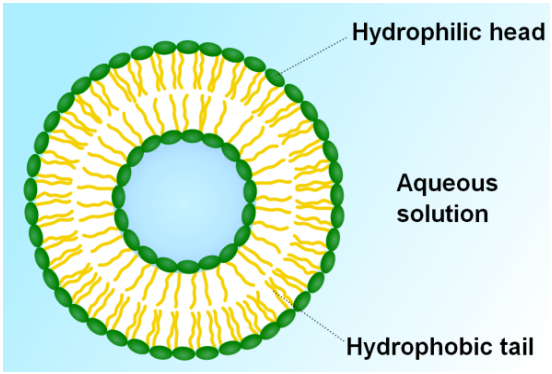
Typically, the cationic lipid molecules are amphiphilic or amphipathic in nature and are made up of three parts: a cationic head-group, a hydrophobic anchor, and a linker (Fig. 12b). The cationic head-group is required for binding and complexation of nucleic phosphate groups, whereas hydrophobic part probably assists in assembling the lipids into polycationic scaffold and facilitating absorptive endocytosis or fusion with plasma membranes [248]. The cationic lipids are classified on the basis of number of positive charges, nature of linker bond, and nature of hydrophobic anchor [248]. Although cationic lipids are the most common lipids used for liposome based transfections, often neutral and/or anionic lipids are mixed with these in variable ratios to neutralize excessive cationic charge and to improve endosomal escape [249]. The anionic lipids when added to nucleic acid-cationic liposome complexes, not only reduce cellular toxicity but also reduce nonspecific interaction with anionic serum proteins like albumin as well as extracellular matrix [250, 251]. Mastrobattista *et al*. (2001) were able to improve transfection by preparing positively charged polyplexes coated with an anionic lipid [251].

The neutral lipids play a role of a helper when formulated with cationic lipids for preparation of liposomes. There are three neutral lipids which are often incorporated in the formulations: dioleyl phosphatidylethanolamine (DOPE), cholesterol and dioleoyl phosphatidyl choline (DOPC). The DOPE is known to destabilize lipid bilayers and is believed to be involved in endosomal disruption and thus enabling nucleic acids to escape endosomes before being destroyed by lysosomes [252]. Cholesterol is also used as a helper lipid. Although it forms more stable but less efficient complexes with nucleic acids than those containing DOPE *in vitro*, cholesterol containing lipoplexes show higher biological activity due to greater cell uptake and stability in serum, compared to lipoplexes with DOPE when these complexes are utilized *in vivo* [248]. The liposome mediated transfection has been shown to take place mostly by endocytosis [236]. However, probably a minor pathway mediated by fusion between siRNA containing lipoplexes and the plasma membrane is responsible for around 5% of siRNA delivery in to cell cytoplasm [236].

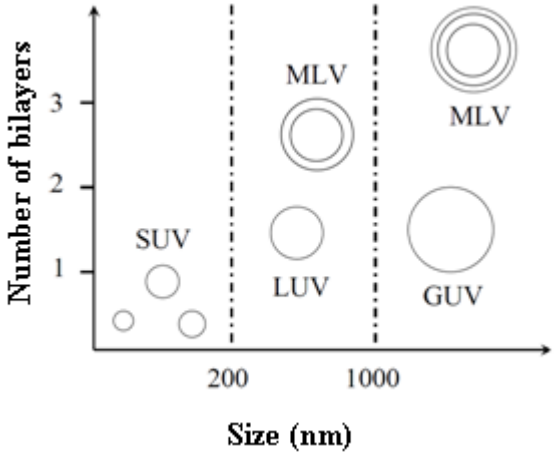
3.2.2.1.1 Cationic liposomes

Liposomes are vesicles composed of a phospholipid bilayers with an aqueous core [253]. They are classified according to their size and their number of bilayers: Small Unilamellar Vesicles (SUV), Large Unilamellar Vesicles (LUV), Multilamellar Vesicles (MLV), and Giant Unilamellar Vesicles (GUV) (Fig. 12b) [254]. The complex formation between anionic phosphate group from nucleic acids and cationic amine head group of cationic liposomes results in formation of lipoplexes, which are quite different from liposomes in structure as the final charge as well particle size changes and these changes are determined largely by lipid / nucleic acid (+ / -) ratios [255].

a



b



c

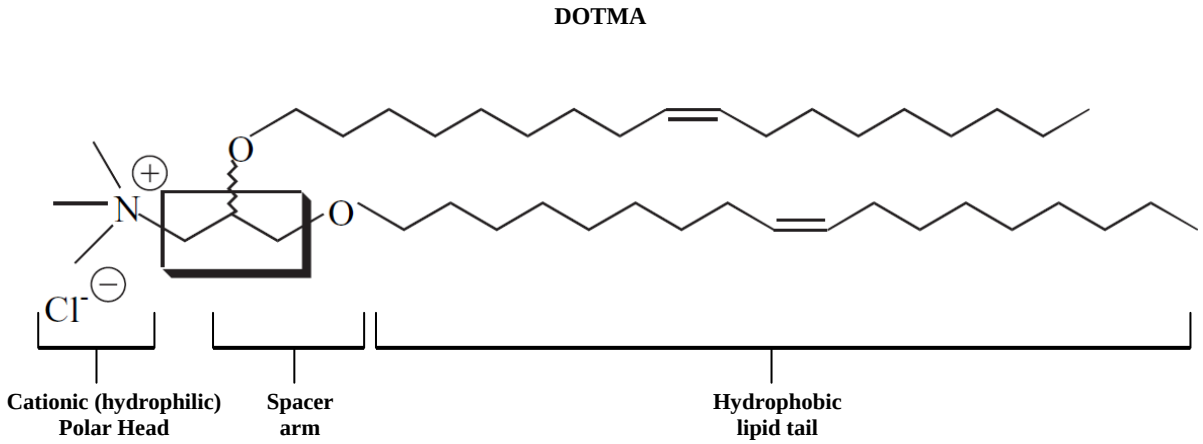


Figure 12: Structure of a cationic liposome. (a) formed by phospholipids in an aqueous medium [256]; (b) Classification of liposomes according to size and number of bilayers [254]; (c) Representative structure of cationic lipid DOTMA modified [257]

3.2.2.1.2 Liposome preparation

The liposomes can be prepared by numerous methods, however the basic principle of their formation involves hydrophilic/hydrophobic interactions between lipid-lipid and lipid-water molecules [258]. Moreover, there is always a need for energy input (sonication, homogenisation, shaking, etc) which promotes the arrangement of lipid molecules in bilayers and achieves a thermodynamic equilibrium in the aqueous phase [258]. The classical method of liposome preparation used by Bangham *et al*. (1965), consists in evaporing the organic solvent into which lipids are dissolved, and then adding an aqueous solvent [241]. The hydration of lipid film causes phospholipids to self-associate so as to hide their hydrophobic chains to the aqueous solvent. It results in formation of bilayers enclosing the aqueous solvent.

3.2.2.1.3 *In vivo* delivery of siRNA with liposomes

DOTAP (N-[1-(2,3-dioleoyloxy)]-N-N-N trimethyl ammonium propane) and Oligofectamine were some of the first lipid formulations used for *in vivo* delivery of siRNA and effective gene silencing of TNF-alpha and beta-catenin in mice [259, 260]. Since then siRNAs have been successfully delivered locally but there are not many reports of successful systemic liposome based delivery of siRNAs against systemic viral infections. However, there are some studies showing promising results in terms of successful delivery of siRNAs and effective viral suppression *in vivo* by formulations based up on neutral lipids. For example, Morrissey *et al*. (2005) using three daily intravenous injections in mice of siRNA (3 mg/kg/day) complexed with the neutral liposomes called "stable nucleic acid-lipid particles" (SNALPs) stabilized with PEG (polyethylene glycol) , could reduce serum DNA of hepatitis B virus by more than 1.0 $\log_{10}$ and the effect lasted for up to 7 days after dosing [20]. In another experiment using intraperitoneal delivery of siRNAs complexed with SNALPs for seven days against Ebola virus has been shown to protect guinea pigs against viremia and death shortly after the virus challenge [261]. Using a lactosylated liposome based upon neutral lipid the phosphatidylcholine, Watanabe *et al*., (2007) could effectively deliver siRNA targeting hepatitis C virus (HCV) in a transgenic murine model, resulting in suppression of intrahepatic HCV expression without interferon response [21]. SNALPs developed by Protiva Biotherapeutics and Alnaylam, have also been successfully used to deliver siRNA in non human primates, whereby using a SNALP-formulated siRNA dose of 2.5 mg/kg, could markedly suppress the Apolipoprotein B (APOB) [262]. Despite encouraging results in murine models, there are no known reports of non-viral delivery of siRNAs in farm animals.

Systemic application of siRNA molecules by lipid based carriers is still challenging and issues of toxicity and need for targeted delivery still need to be addressed.

3.2.2.2 Cell penetrating peptides (CPPs)

CPPs or protein transduction domains (PTDs) are short peptides having fewer than 30 residue peptides, derived from natural or unnatural protein or chimeric sequences [263]. Most of the CPPs possess a high density basic amino acids (arginines and/or lysines), which are proposed to interact with the anionic surface of the cell membrane and enhance internalization of the peptides [264]. Among chemical delivery vectors CPPs are unique in that the concentrations of CPPs that are used for molecular delivery, produce very low or undetectable cytotoxicity [264].

CPPs were first discovered when Frank and Pabo in 1988 observed that HIV-1 Trans-Activator of Transcription (Tat) protein, which transactivates transcription of the HIV-1 genome, crossed the cell membrane by itself [265]. Later, a minimal peptide fragment of Tat (49-59 amino acids) involved in cellular uptake was identified by a French group [266]). The Tat peptide has been shown to successfully deliver siRNAs *in vitro* [267]. Another major discovery in CPP domain was made by Joliot *et al* (1991), who demonstrated that Drosophila Antennapedia home domain could be internalized by neuronal cells [268]. This work lead to discovery of the first CPP derived from a non-viral protein which consisted of a 16-mer peptide derived from the third helix of the Drosophila Antennapedia homeodomain protein and was named Penetratin^{TM} [269]. The Penetratin is capable of transfecting nucleic acids antisense oligonucleotides [270], peptide nucleic acids (PNAs) [271]. A MPG peptide was used to perform the first non-covalent delivery of nucleic acids in 1997 [272]. The MPG peptide is an artificially constructed 27 amino acid amphipathic CPP containing a lysine rich basic region derived from the nuclear localization signal (NLS) of the SV40 large T antigen and a hydrophobic region derived from the HIV-gp41 coat protein [272]. Both the MPG peptide and a modified MPGΔ^{NLS} (having a single mutation of the second lysine residue in the NLS motif to serine) can transfect siRNA *in vitro* however latter is more efficient as it delivers siRNA into cytoplasm instead of nucleus [273]. MPG peptides have been also used to deliver siRNA into mice by systemic route [274]. Another milestone was achieved when a chimeric CPP "Transportan", derived from the N-terminal fragment of the neuropeptide galanin linked to a wasp venom peptide called mastoparan was successfully used *in vivo* for delivery of peptide nucleic acids (PNAs), in mice [271]. The first CPP based transfection of siRNA was performed with a MPG peptide [275] and since then numerous natural and as well

as engineered CPPs have been tested for siRNA delivery [276]. Polyarginine-based peptides have also been successfully used for siRNA delivery. Majority of the CPPs possess basic amino acids (arginines and/or lysines), through which these interact with cell membranes and help in the internalization of CPPs [264]. The experiments on Tat and penetratin revealed that the role of positive charges is crucial for translocation [277]. After studying various cationic polypeptides, CPPs with polyarginines were found to be more efficient than other cationic polypeptides like polyhistidines and polylysines and among polyarginine peptides, Arg7 and Arg9 have been the most widely used for *in vitro* and *in vivo* delivery [277].

Although cellular uptake mechanism for CPPs has been reported to be associated with endosomal pathway [278], there is no unified mechanism established for CPP uptake mechanism up to now, and probably numerous factors are involved [263]. However, there is a consensus that the initial contact between the CPPs and the cell membrane take place by electrostatic interactions with negatively charged proteoglycans [263]. Moreover, as CPPs are diverse in chemical and physical structures, it is suggested that different properties like CPP molecule length, charge delocalization as well as size and charge of the cargo can have impact upon peptide uptake mechanism [279]. Cellular uptake of CPPs, therefore, does not occur by any single mechanism thus, energy independent direct diffusion, macropinocytosis, clathrin-mediated endocytosis, and caveloe/lipid raft mediated endocytosis may all occur [280].

CPPs can be classified in two ways. These are classified either on the basis of mode of bonding with cargo into, those requiring covalent linkage with siRNAs and others which, being amphipathic, can form stable non-covalent bonds. Secondly, these can also be subdivided structurally into those which polycationic having clusters of polyarginine in the primary sequence or those which are amphipathic [263].

3.2.2.2.1 Covalent attachment of CPP to siRNA

Tat conjugated to the modified antisense strand of siRNA was successfully used to inhibit EGFP gene *in vitro* [281]. However, most of Tat-siRNA was found localized in endosomes and there was a concern that the attachment of Tat to siRNA may disrupt functionality of siRNA molecules [282]. To address this concern, CPPs have also been linked to siRNA through disulfide bond which is cleaved when conjugates reach reducing environment of cytosol, resulting in successful RNAi [283, 284]. The covalent strategy usually requires

complex chemistry for conjugation and there is also risk of alteration of biological activity of siRNA therefore non-covalent strategy of delivery appears more suitable [263].

3.2.2.2.2 Non-covalent CPP-siRNA complex formation

Assembling siRNA/CPP complexes through non-covalent interactions is advantageous as it simplifies conjugation protocols but also eliminates the need for optimization of individual syntheses schemes. Additionally, there is also lower likelihood that CPP will interfere with the bioactivity of the cargo [263]. The non-covalent delivery of siRNA is based up on electrostatic interactions between siRNA and amphipathic peptides [264]. The amphipathic peptides possess hydrophobic (polar) and hydrophilic (non-polar) domains. The amphipathic property of CPPs may arise from either primary or secondary structure [277]. Primary amphipathic peptides consists of the sequential assembly of a domain of hydrophobic residues with a domain of hydrophilic residues while secondary amphipathic peptides are produced by conformational state that allows positioning of hydrophobic and hydrophilic residues on opposite sides of the molecule [277].

Since siRNAs are negatively charged, they can bind with positively charged CPPs through nonspecific electrostatic interactions and provide permeability by covering the siRNA surface with positive charges from the CPP [264]. In 2003, a non-covalent strategy based on MPG was found to efficiently deliver siRNA into cell lines [285]. Similarly, this non-covalent mode of delivery has also been used for other CPPs like, Tat, polyarginine, and transportan-derived peptides [263]. A novel 20-amino acid amphipathic peptide, CADY, has been recently described which combines both cationic arginine and aromatic tryptophan residues into its design [286]. It forms stable complexes with siRNA through electrostatic interactions and interestingly uses a non-endocytic mechanism to pass through plasma membrane, thus avoiding endosomal entrapment [286]. This CPP can transfect a variety of cell lines, including difficult to transfect primary cell lines [286]. Since CADY-siRNA is stable in 50% serum for 24 hours and leads to significant knockdown with sub-nanomolar concentration, it may be effective *in vivo* as well [286].

3.2.2.2.3 *In vivo* delivery with CPPs

CPPs have been delivered *in vivo* with some successes and failures. MPG peptide was used for systemic *in vivo* delivery of siRNA targeting essential cell cycle protein cyclin B1, resulting in efficient blockage of tumor growth [287]. In another experiment, siRNA targeting HIV was successfully delivered through a CD7-specific single-chain antibody conjugated to

the oligoarginine peptide (scFvCD7-9R) could effectively suppress HIV infection in a mouse model [22]. The CD7 receptor is rapidly internalized after antibody binding, therefore it has been exploited for the targeted delivery of several monoclonal antibody (mAb) [22]. Kumar *et al.,* [27] using a nonamer arginine was introduced at the carboxy terminus of a peptide derived from rabies virus glycoprotein (RVG) were able to transfer siRNA into neuronal cells *in vivo*, resulting in efficient gene silencing after intravenous injection into mice. Furthermore, systemically delivering of RVGR9 conjugated antiviral siRNA complex could protect mice from encephalitis induced by Japanese encephalitis virus (JEV) infection, which is the first report on a nontoxic method to deliver siRNA across the blood brain barrier [288]. CPP based siRNA delivery has also entered at level of preclinical and clinical trials, with Traversa Inc., are testing HIV Tat-based and Panomics Inc., testing secondary amphipathic peptide-based non-covalent delivery of siRNA [263].

3.2.2.3 Other chemical vectors

Aptamers are RNA or DNA oligonucleotides that fold by intramolecular interaction into unique three-dimensional conformations capable of binding to target antigens with high affinity and specificity [289]. The aptamers have been used as siRNA vectors in a mouse tumor xenograft model, however their systemic use requires addition of nuclease stabilizing agents and endosmolytic functionalities [18]. Dendrimers molecules are repeated branched species characterized by structural perfection [18]. Dendrimers have been used successfully for *in vitro* delivery of siRNAs [290]. Polyethyleneimines (PEI) are polycation-containing block copolymers like [291], which have also been used for delivery of siRNA molecules *in vivo*, although PEI use *in vivo* has some toxicity issues [292]. Chitosan is a biodegradable, biocompatible and non-toxic cationic polymer obtained from deacetylation of chitin, which have been proposed as biocompatible alternative cationic polymers, suitable for nonviral nucleic acid delivery[293]. Chitosan have been used for siRNA delivery *in vitro* as well as *in vivo* [294]. Antibody-protamine fusion carriers have also been shown to be efficient in delivery of siRNA to HIV-infected or envelope-transfected leukocytes [295].

3.2.3 Viral vectors

Like chemical vectors, the viral vector based delivery of shRNA also profited from technology that already existed for gene therapy. Soon after delivery of siRNAs *in vitro* [175], it was demonstrated that siRNAs can also be expressed from plasmid DNA as “short hairpin RNAs” (shRNAs) [296, 297]. This finding paved the way for viral vector based RNAi

therapeutics. U6 [297] and H1 [296] were the first polymerase III promoters described that express functional siRNAs [298]. However U6 promoters have been reported to be more efficient *in vivo* [299]. Typically, shRNA transcription starts in a position outside the promoter sequence, continues along 19-29 nucleotide long top strand, the 4-19 nucleotide long hairpin loop and finally the bottom strand and terminates after the second or third residue of track of 4-6 thymidines [298]. The termination is so designed that it results in a 3' two-nucleotide overhang after RNAse III cleavage as is the case with natural pre-miRNAs [298]. The shRNA sequences are selected and translated to DNA, and these are normally synthesized in the form of two complimentary oligonucleotides that are annealed and cloned downstream of the selected promoter and regulatory sequences [298]. The constructed shuttle plasmids expressing shRNA are next tested for inhibition of target gene *in vitro*. Moreover, the cassettes may be inserted into expression clones for production of required recombinant virus vectors. In addition to standard shRNA resulting in production of a 21 nt long siRNAs, numerous other variations of shRNA have also been used. Longer shRNAs producing 27- 29 nt long siRNAs [300, 301], and multiple shRNAs simultaneously expressing different shRNAs against different target regions have been used [302, 303]. The viral vectors have certain advantages over chemical vectors for delivery of siRNAs like [298];

1. tissue specific delivery of shRNAs.
2. possibility of inducible or tissue specific promoters.
3. both transient as well as stable expression of shRNA is possible, according to the viral vector chosen.
4. better transduction efficiency compared to non-viral transfections even in cells like lymphocytes that are traditionally hard to transduce.
5. cost effectiveness.
6. most of the available viral vectors have already been tested clinically in phase I safety trials.

The most widely used viral vectors for shRNA delivery include adenovirus, adeno associated virus (AAV), lentivirus, retrovirus, baculovirus. In contrast to the gene therapy, shRNA expressing cassettes tend to be of small length and their expression is possible even by the smallest vectors [298]. However, viral vectors are selected on the basis of tissues required to be transduced and also whether stable or transient transduction is required. For chronic infections like HIV stable transduction is desirable to avoid repeated administration of vectors while for acute infections, transient transductions with shRNA expressing vectors would suffice. The RNA viruses such as retrovirus and lentivirus produce stable transduction as they

integrate into host genomes while non-integrating DNA viruses like adenovirus, baculovirus and AAV maintain their genome episomally in the host cells.

3.2.3.1 Adenovirus vectors

Adenoviruses (Ads) are non-enveloped viruses having a linear double stranded 36 kbp DNA genome with non-enveloped icosahedral protein capsid. Adenoviral capsids are made up of three major protein components – the hexon, penton base and fiber (Fig. 13) [304]. In general, the fiber knob functions as the major attachment site for cellular receptors, while the penton base is involved in secondary interactions that are required for virus entry into the cell [304]. More than 50 serotypes have been identified which are divided into six subgroups (A-F). Out of the numerous serotypes, Ad2 and Ad5 belonging to subgroup C were first shown to serve as efficient vectors gene delivery *in vitro* [305] and still are the most commonly used adenoviral vectors [306]. For gene therapy as well as for RNAi, replication defective adenoviruses can be used since they are non-pathogenic and can transiently transduced a variety of both dividing and non-dividing cells without integration to the host genome [298].

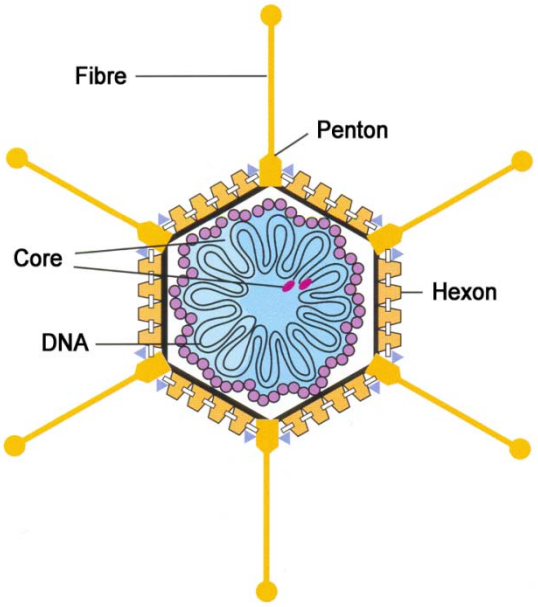


Figure 13: Structure of Adenovirus [307].

In the past it has been considered that adenovirus serotypes belonging to species A, C, D, E and F use the coxsackie and adenovirus receptor (CAR) as a docking site providing a high affinity virus-to-host association [308]. CAR was previously thought to be a primary Ad5 receptor, but now there are studies according to which adenovirus type 5 also uses many other receptors for docking like the heparan sulfate proteoglycan (HSPG), vascular cell adhesion molecule 1 (VCAM-1), major histocompatibility complex class I (MHC 1), scavenger

receptors (SR), while indirect binding it also uses dipalmitoyl phosphatidylcholine receptors (DPPCRs), coagulation factor X (FXs), and Lactoferrin receptors (LfRs) [309]. Although receptor binding is thought to play a major role in adenoviral tropism, this alone cannot explain all aspects of *in vivo* host-virus interactions like enhanced transductions of liver cells or adenoviral uptake by Kupffer cells [310]. Excessive adenoviral binding to hepatocytes may be partly explained by binding of central depression of adenoviral hexon with Gla domain of coagulation factor X (FX) which has been found to produce efficient transduction of hepatocytes [311]. That may explain excessive liver tropism of adenovirus where FX could be the main determinant of *in vivo* liver transduction [311].

The internalization of adenovirus occurs by a secondary interaction between RGD motifs on penton base protein and integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [312] through clathrin-coated pits mediated endocytosis [313]. The virus then escapes by lysing endosomal membrane and with the help of microtubule mediated translocation, enter the nuclear pore complex where viral DNA is released for expression [314]. The transcription of wild type virus initiates with expression of early E1 genes, which activate synthesis of viral genes and replication. E1 and the non-essential E3 genes were deleted in first generation adenoviral vectors to render them replication deficient [298]. But these first-generation adenoviruses express viral genes to low levels, including virus associated (VA) RNAs which have been described to saturate the cellular silencing machinery, leading to toxicity [315]. Although functional inhibitory exogenous shRNAs have been expressed for first generation adenoviral vectors without saturation of silencing machinery [316]. The second generation vectors, which have deletions in the E1-E4 locus are comparatively less immunogenic and show prolonged expression of recombinant genes [317]. Whereas third generation adenoviruses are produced by deleting all the viral genes, as a result of which expression of viral genes is avoided resulting in lesser immunogenicity [318].

The recombinant replication deficient adenoviruses have been successfully used *in vitro* as shRNA delivery vectors against numerous viruses like hepatitis B virus [221], measles virus [319] and hepatitis C virus [320]. The adenoviral vectors expressing shRNA have been successfully used *in vivo* against several viral infections. After pre-treatment of guinea pigs and swine with adenoviral vectors expressing shRNA against foot and mouth disease virus (FMDV), and challenging 24 hours later with the target virus, Chen *et al.,* could protect the animals from major clinical manifestation of the disease [23]. Similarly adenoviral vectors have also been used to protect mice against porcine circovirus type 2 [225].

3.2.3.2 Baculovirus vectors

The baculoviruses belong to the family *Baculoviridae* which is subdivided into two genera: the granuloviruses (GV) and nucleopolyhedroviruses (NPV). The GV contain one nucleocapsid per envelop but the NPV may either contain single (SNPV) or multiple (MNPV) nucleocapsids for each envelop in the occlusion body [321]. Furthermore, the NPV capsids are occluded in polyhedron matrix and the polyhedra may contain multiple embedded virions [322]. Among the numerous baculoviruses, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the most well studied and extensively used virus in foreign gene expression [323]. AcMNPV possesses a circular double stranded DNA genome which is condensed into a nucleocapsid core by a protamine-like protein. Naturally AcMNPV are occluded in a polyhedron which after being ingested by insects is dissolved in alkaline midgut, releasing infectious virions [323].

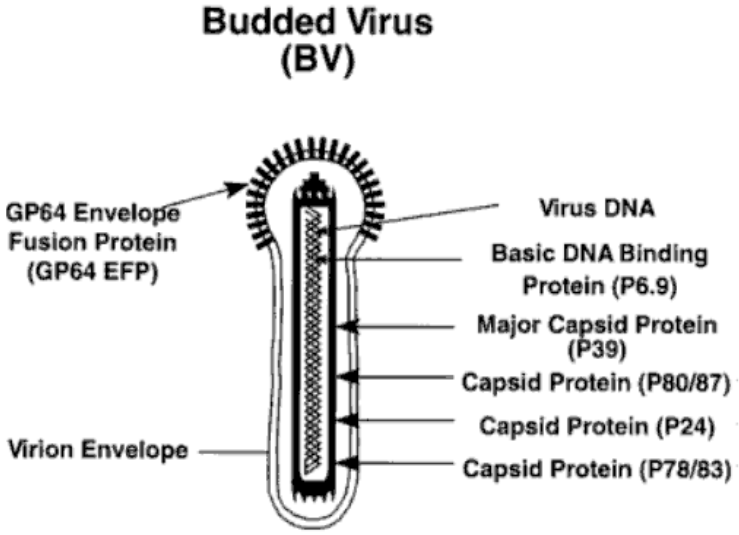


Figure 14: Structure of a Budded baculovirus. According to Blissard, 1996. [324]

The AcMNPVs only replicate in insect cells and naturally infect insects belonging to the order *Lepidoptera*. Baculoviruses primarily enter insect cells through clathrin-mediated, low-pH dependent endocytic pathway while they may enter mammalian cells through multiple pathways including caveola-dependent mechanism as well [325]. However, AcMNPV can infect insect cells and transduce mammalian cells even in the absence of endocytosis, by direct fusion at cell surface under low pH conditions [326].

The baculoviruses can enter the mammalian cells but they are unable to express their genes because the baculovirus promoters are inactive in these cells [327]. One of the interesting consequences of this is the absence of pre-existing antibodies against baculovirus in mammals [327, 328]. For gene transfer and expression in mammalian cells, so-called BacMam viruses

have been generated by incorporation of mammalian cell-active expression cassettes [329]. BacMam baculoviruses are capable of transducing wide variety of cells including non-dividing cells [330] and primary cells [331]. They have been used to reduce viral infections *in vitro* of numerous viruses like porcine arterivirus [332], hepatitis C virus [333], hepatitis B virus [334], and influenza viruses A and B [335].

The baculovirus can prove to be good shRNA delivery vectors *in vivo*. Although delivery of siRNA to the appropriate cells or tissues is a major challenge, problem of inactivation by complement is resolved [336]. Chemical or genetic modification can also overcome problem of serum inactivation [337, 338]. Furthermore, it is reported that baculoviruses do not have deleterious effects on mammalian cells even when used at very high multiplicity of infections (MOIs) [339]. Moreover, not only can the baculoviruses be easy and cheap to produce, they can be grown to high titers in cell cultures as well. Although baculovirus vectors have not been used thus far for shRNA delivery *in vivo*, but these have been successfully used for gene transfer into mouse brain and rabbit retina [340, 341].

3.2.2.3 Other viral vectors

Many other viral vectors have been used for RNAi mediated silencing of viral infections like adeno-associated virus (AAV) [342], herpes simplex virus [24], retroviruses [296], and lentiviruses [177]. Moreover, these viral vectors have also been used against infectious disease of veterinary importance. Infectious bursal disease virus (IBDV) by an avian adeno associated virus delivered miRNAs targeting VP1 and VP2 genes *in vitro* [343]. Recombinant herpesvirus of turkey (HVT) expressing shRNAs against genes gB and UL29 of the Marek's disease virus (MDV) moderately reduced viremia in chicken [24]. In another study, avian leukosis virus-based retroviral vectors expressing shRNA against MDV gB glycoprotein gene and ICP4 transcriptional regulatory gene could significantly reduce MDV viremia *in vivo* [25].

4. Control of Morbillivirus Replication by RNAi – State of the art

RNA interference has become the method of choice for suppression of gene expression *in vitro*. The synthetic short interfering RNAs (siRNAs) represent a new class of molecules with a significant potential for therapeutic applications. This technology promises to bring benefits in this field that far surpass the antivirals currently available in the market, as it offers new possibilities to easily design highly selective antiviral drugs capable of targeting specific genes in order to suppress its expression and this with a very low toxicity as its natural nucleotide components are easily metabolized by cellular systems [344].

This method is now emerging as a powerful tool for research with many published studies using siRNA in mammalian cell culture. They show that synthetic siRNA are particularly effective in the areas of oncology and the treatment of viral infections. siRNAs have been widely used against viruses of various families to inhibit the intracellular viral life cycle. The first experiments carried out on an unsegmented, negative sense, RNA virus, the respiratory syncytial virus (RSV), go back to 2001 [175]. A publication by Reuter *et al*., [345], describes the generation of multiple siRNAs directed against 6 genes of a morbillivirus, measles virus (MV) can effectively deregulate gene expression in general with siRNAs against transcripts of the nucleocapsid (N), phosphoprotein (P) and polymerase (L), which form ribonucleoprotein (RNP) complex. Two other publications describe the use of siRNA to inhibit replication in cell culture of MV and subacute sclerosing panencephalitis (SSPE) virus [346, 347]. However, these studies show inhibition of measles replication only *in vitro* but siRNA against morbilliviruses have not been successfully delivered *in vivo* so far.

In the case of morbilliviruses, despite existence of efficient vaccines against morbillivirus diseases, no effective treatments exist for infected animals or humans. RNA interference (RNAi) has a potential of being turned into an effective and specific antiviral therapy for the control of these diseases. In case of disease outbreaks, the emergency vaccination alone, in poor countries does not prevent heavy economic losses arising from animal mortality as well as morbidity. Moreover, even after administration, the vaccines require several days before a sufficiently strong protective immune response is present. Under these circumstances, an antiviral that can block viral replication in the early stages of infection could be an important complementary tool in the control strategy. A combination of antiviral agent together with vaccination may reduce the economic losses incurred during an epidemic.

The *in vitro* tests conducted by the CIRAD have helped in identification of three sites susceptible to RNAi, which are located on the conserved regions of nucleoprotein gene of the morbilliviruses, through an siRNA design methodology that allows the rapid prediction of potential sequences on target mRNAs [348]. These strategies for predicting design done on the conserved regions of the genome should lead to the use of lower doses of siRNA while reducing non-specific off-target effects. Indeed, knowledge of morbillivirus genomes allowed us to develop an antiviral approach based upon RNAi against these three morbilliviruses. Briefly, sixty two 19 nt long siRNA sequences targeting N, M, and F genes of three genes of peste des petits ruminants virus (PPRV), rinderpest virus (RPV) and MV were designed, chemically synthesized and tested for efficacy *in vitro* [348-350]. Three sequences directed against nucleoprotein (N) gene, namely NPPRV1, NPPRV6, and NPPRV7, were found to be most effective and resulted in reduction of viral titer to about 1,000 to 10,000 times and also an inhibition of up to 90% of the nucleoprotein expression by PPRV when transfected at a final concentration of 100 nM (Tab. 1).

However, for systemic administration of siRNA *in vivo* to be successful, numerous constraints, as were described in the review of literature of this document, including poor transfection *in vivo*, which is also the major issue with other nucleic acid based strategies, a low serum stability and toxicity need to be overcome. Even after more than a decade of discovery of RNAi and despite excellent knock down of viral or cancer genes *in vitro*, its therapeutic application has not been possible due to these difficulties involved in the efficient *in vivo* delivery. Although some siRNA based therapeutics have entered clinical trials, these are mostly for local use only, whereas systemic delivery has proven to be a significant hurdle. Since PPR is an acute and systemic infection of small ruminants, transient transfection or transduction are required for systemic delivery of siRNA/shRNA *in vivo*. For that purpose, in this work several chemical delivery vehicles as well as viral vectors were considered for their ability to deliver the siRNA NPPRV1 directed against the nucleoprotein gene of PPRV, previously selected as the most effective in inhibiting PPRV replication after delivery by a commercial liposome in cell cultures.

A process of reflection by the laboratory, on the selection and development of delivery vehicles was crucial for the success of *in vivo* studies. Indeed, due to their intrinsic mechanisms, the viruses prove efficient delivery vectors, while despite certain drawbacks like toxicity, the cationic lipids are also delivery systems of choice. Thus the laboratory had therefore developed a replication-deficient human adenovirus type 5 expressing shRNA

NPPRV1 (rAd_NPPRV1^{shRNA}) against the nucleoprotein of PPRV and tested it *in vitro* (personal communication), showing it was capable of reducing PPRV induced cytopathic effects (CPE) as well as PPRV titers (Fig.15).

Table 1: Inhibition of PPRV N gene expression and PPRV titers by three effective siRNAs

siRNA name	siRNA Sequence	Position	Fold Inhibition of PPRV Titer	% Inhibition of NPPRV expression
NPPRV1	Sense 5′-GGAUCAACUGGUUUGAGAAtt-3′	480-498	10,000	90+- 2
NPPRV6	Sense 5′- GGCGGUUCAUGGUAUCUCUtt-3′	741-759	100	>80
NPPRV7	sense 5′-GCAUUAGGCCUUCACGAGUtt-3′	899-917	100	>80

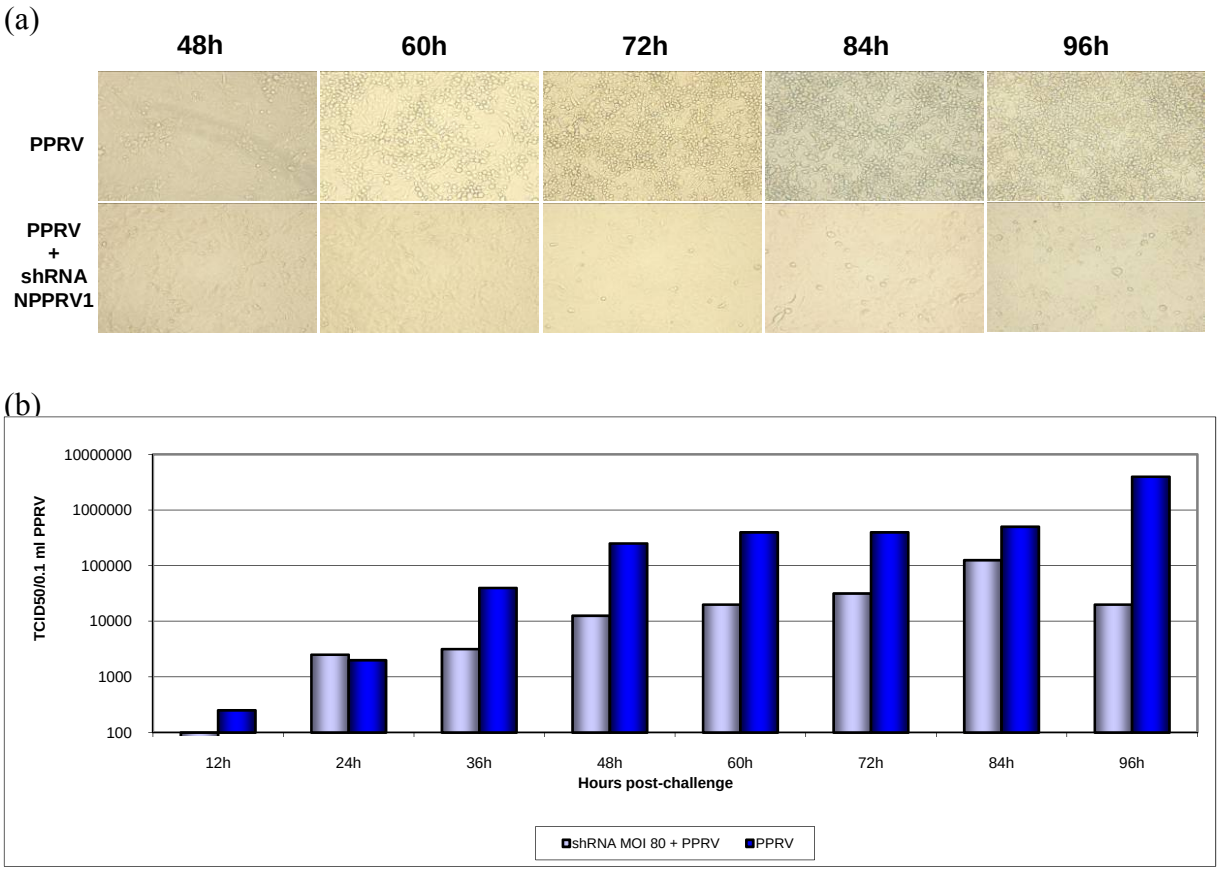


Figure 15: (a) Inhibition of cytopathic effects (CPE) in Vero cells, and (b) PPRV progeny by rAd_NPPRV^{shRNA}, at various time intervals after challenge with PPRV MOI 0.1.

AIMS AND OBJECTIVES

The aim of this project was the effective delivery of siRNA or shRNA *in vivo* against peste des petits ruminants virus. For the purpose, firstly the viral and chemical vectors were tested in the course of study to find efficient delivery vectors *in vitro*. The *in vivo* experiments were initially conducted on goats under L3 bio-security conditions. However, due to ethical, economical, and bio-security issues, a mouse model based upon bioluminescent imagery is developed for *in vivo* testing of various siRNA/shRNA delivery vectors.

PART. 1:

In vivo delivery of siRNA/shRNA against PPRV infection by adenoviral and cationic liposome vectors

1.1 Introduction

RNAi based antivirals can be administered *in vivo* either in the form of chemically synthesized siRNA molecules which are delivered by chemical vectors or these can be delivered in the form of shRNA through viral vectors (for further information, see Review of the Literature chapter). At the start of this work, siRNAs capable of effective inhibition of PPRV replication *in vitro* were available. Since commercial chemical vectors prove too expensive when used for *in vivo* delivery of large quantities of siRNAs in animals of large size like goats, here a cationic lipid based formulation was developed for delivery of siRNAs. In this study, this cationic lipid as well as a recombinant adenovirus expressing the shRNA NPPRV1 (rAd_NPPRV1^{shRNA}) were tested about their capacity to deliver the siRNA/shRNA NPPRV1 and inhibit PPRV replication in goats.

1.2 Material and Methods

1.2.1 Cell culture

Vero cells (European Collection of Cell Culture, France 84113001) were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% (vol/vol) heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine at 37°C with 5% CO2 in a humidified incubator. Vero cells were used for the production and titration of PPRV and transfection experiments. The 293A cells (Invitrogen) were propagated in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) FBS at 37°C with 5% CO2.

1.2.2 Production of PPRV

The vaccine strain of PPRV (Nigeria 75/1) [351] and a virulent strain of PPRV Côte d'Ivoire 89 (CI 89) were propagated by infecting Vero cells, cultured in 75 cm^2 cell culture flasks with EMEM having 5% FBS at multiplicity of infection (MOI) of 0.1 $CCID_{50}$/ml. When viral cytopathic effect (CPE) was almost complete (~80%), the cells were freeze-thawed 3 times and aliquots were stored at -80°C. Viral titration was performed in 96 well cell culture plates, in which 100 µl of 10 fold serial dilutions of PPRV in EMEM were added to 100 µl of Vero cell suspension (20,000 cells). For each viral dilution, 10 wells were used. The viral titers were calculated by end point titration method of Reed and Muench [352] and expressed as cell culture infectious cytopathic doses for 50% of the tests ($CCID_{50}$ /ml).

1.2.3 Preparation of liposomes for *in vitro* validation of siRNA delivery

A liposome formulation (M2b) based up on a 1/1/0.5 (m/m) combination of cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane-chloride salt (DOTAP), the neutral lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and an anionic lipid cardiolipin was developed and tested *in vitro* for delivery of siRNA NPPRV1. DOTAP, DOPE, and cardiolipin were each dissolved in ethanol (90%) to have a concentration of 25µg/µl. Then, 20µl each of DOTAP and DOPE were mixed in ratio of 1/1 (m/m). Solution was homogenized by vortexing for 10 seconds and incubated at room temperature for 15 minutes. The tube containing the lipid mixtures was vacuum centrifuged overnight at room temperature to obtain a dry lipid film. Then, 10 µl of cardiolipin was added to the tube with lipid film and vortexed until film was completely dissolved. Next, 190 µl of sterile water (filtered by 0.2 µm) was rapidly added with pipette, in a single step, under hood and vortexed immediately for 10 seconds. The solution was incubated overnight at 4^{o}C before using the liposomes for making complexes.

1.2.3.1 Transfection *in vitro* of siRNA with cationic liposomes and challenge with PPRV

Vero cells of 48 hours of growth were trypsinized and 10^5 cells were cultured in 24 well cell culture plates using EMEM supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine at 37°C with 5% CO2 in a humidified incubator. At the time of transfections, cell layers were more than 80% confluent. Before use, liposome solution (6.25 mg/ml) was homogenized by light vortexing and placed at room temperature for 15 minutes. Next, it was used either undiluted for high siRNA/lipid mass ratios (1/90, 1/70, and 1/56) or diluted 1/9 in NaCl (150 mM, pH 5.2) for lower siRNA/lipid ratios (1/12.4, 1/10, and 1/6.2) to have a concentration of 0.694 mg/ml.

The synthetic siRNAs, NPPRV1 which targets N gene of PPRV and the irrelevant siRNA NRPV1 [3] targeting N gene of rinderpest virus, were used for transfection. For complex formation, 0.41 µg of each siRNA was diluted to a final volume of 45 µl/well in RNAse free water. The liposomes were diluted variably (according to variable mass ratios to be used against siRNA) in NaCl solution to a final volume of 45 µl/well and mixed. For lipoplex formation, the siRNA solution was rapidly injected upon liposome solution in one step, pipetted three times, and lightly vortexed. The complexes were allowed to form at room temperature for 30 minutes. Finally, 90 µl of complexes were added to each well of 24 well cell culture plates, already having 210 µl of EMEM, resulting in a final siRNA concentration of 100 nM. After five hours of incubation at 37°C and 5% CO2, the complexes were removed

and fresh EMEM supplemented with 5% FBS and glutamine was added to each well. Lipofectamine™ 2000 (Invitrogen) was used as control liposome, according to the supplier's protocol.

Twenty four hours post-transfection, the cells were infected with the PPRV vaccine strain Nigeria 75/1 using a MOI of 0.1 $CCID_{50}$ per cell. For that, cell culture medium was removed and viral inocula were added in EMEM without serum in a final volume of 200 µl in a 24 well cell culture plates. After one hour of incubation at 37°C with 5 % CO2, EMEM with 5% FBS was added to have a final volume of 1 ml per well and plates were again incubated. The positive controls for transfection consisted of the Vero cells transfected with the irrelevant siRNA NRPV1 and infected with the same MOI of PPRV. Moreover, the cells serving as negative controls were similarly transfected but not infected with PPRV. Efficacy of siRNA was assessed on the inhibition of N protein and CPE produced by PPRV compared to the positive control. Efficacy of M2b liposomes was compared to Lipofectamine™ 2000.

1.2.3.2 Measurement of PPRV N protein expression and cytopathic effects (CPE) of PPRV in siRNA transfected cells

The relative expression of PPRV N protein was used as a measure of the N gene knock-down by siRNA NPPRV1 transfected with different liposome formulations. The expression of the N protein was quantified by immunofluorescence staining and reading by flow cytometry 96 hours post-infection, as described by Servan de Almeida *et al*. [353]. To measure the inhibition of CPE, cell layer was checked by microscopy 96 hours after PPRV challenge. Scores ranging from 0% to 100% were attributed depending on the severity of CPE (Fig. 16).

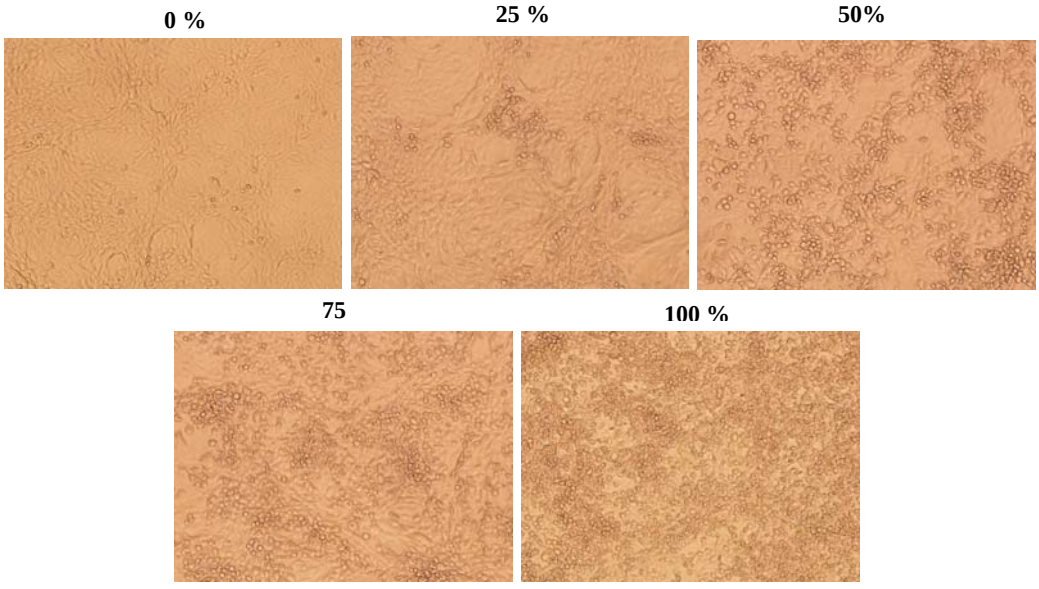


Figure 16: Percentage scale of CPE produced by PPRV on Vero cells.

1.2.4 Construction of recombinant adenovirus rAd_NPPRV1^{shRNA}

Recombinant adenovirus expressing shRNA NPPRV1 (rAd_NPPRV1^{shRNA}) available at the beginning of this work, was constructed using the commercial kit BLOCK-iT^{TM} Adenoviral RNAi Expression System according to the supplier instructions (Invitrogen). Briefly, the double-stranded oligo encoding the shRNA NPPRV1 was cloned into the pENTR^{TM}/U6 plasmid (Invitrogen) encompassing the human U6 promoter, and a polymerase III terminator. Recombination was performed using pENTR/U6/ NPPRV1^{shRNA} clone and pAD/ BLOCK-iT^{TM} –DEST vector to generate an adenovirus expression clone. The expression clone was transfected into 293A cells to produce the rAd_NPPRV1^{shRNA} stock (Fig. 17).

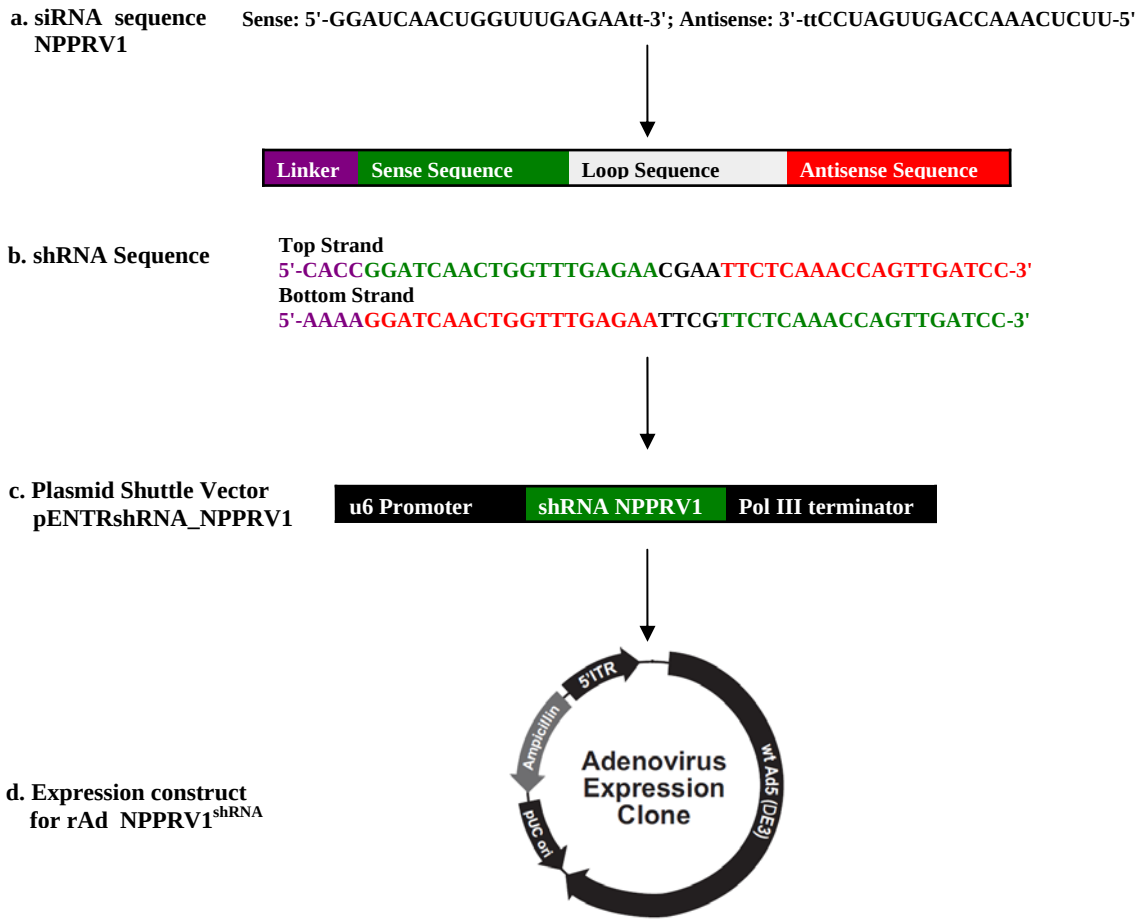


Figure 17: Schematic representation of (a) siRNA NPPRV1 sequence (b) shRNA sequence, (c) plasmid shuttle vector, and (d) expression plasmids for rAd_NPPRV1^{shRNA}.

1.2.5 *In vivo* delivery of siRNA/shRNA by "M2b" liposome and rAd_NPPRV1^{shRNA} vectors

1.2.5.1 Preparation of liposomes and lipoplexes for *in vivo* application

DOTAP, cardiolipin sodium salt from bovine heart, and DOPE were purchased from Avanti Polar Lipids, Inc. Endotoxin free water was used for siRNA dilution as well liposome preparation. An inherent problem occurred in scaling-up experiments from cell cultures to large animals like goats is the sense that larger volumes as well as concentrated reagents had to be used to achieve rational injectable doses. A siRNA/lipid ratio of 1/6.2 was intended to be used for experiment *in vivo* but due to problem of precipitation at this ratio, a ratio of 1/2.08 was retained.

For this preparation, 20 ml of ethanol (90%) were added to 500 mg of cardiolipin lipid and vortexed for one minute. 1 mg of each DOPE and DOTAP powders were diluted in cardiolipin solution resulting in a final lipid concentration of 125 mg/ml. The mixture was then heated in a water bath at 50°C for 1h. Eighty milliliters of water pre-warmed to 50°C was rapidly injected onto 20 ml of dissolved lipids to have a liposome solution in a final lipid concentration of 25 mg/ml. To prepare working solution of the liposomes, original liposome solution (DOTAP+DOPE+cardiolipin 25 mg/ml) was diluted three times in NaCl (0.9%, pH 5.2) to have a final lipid concentration of ~8.33 mg/ml. The solution was vortexed for mixing. The 50 mg lyophilized siRNA NPPRV1 was diluted in endotoxin free water to a final concentration of 2 mg/ml and solution was well mixed.

For lipoplex preparation, liposomes were first extruded three times with a discontinuous extruder (Lipofast-extruder, Avanti Polar Lipids, Inc.) through 100 nm filter. The lipoplexes were prepared using siRNA/lipid mass ratio 1/2.08 (12mg of siRNA for 25 mg of lipids). The complexes were allowed to form at room temperature for 30 minutes. Finally, Dulbecco's Minimum Essential Medium (DMEM) was added to complete 30 ml, which was the volume injected intravenously to the goats.

1.2.5.2 Experimental design

For the *in vivo* experiment, thirty goats were confined in the Experimental Infectiology Platform (PFIE) of INRA (Nouzilly, France). The animals were pre-treated with rAd_SCR^{shRNA}, rAd_NPPRV1^{shRNA} or lipoposomes M2b complexed with siRNA NPPRV1 and subsequentlychallenged with the virulent strain of PPRV - Côte d'Ivoire-89 (CI 89). The

animals were divided in 3 groups of 10 animals each. Group-1 and Group-2 were injected intravenously with $0.5x10^{10}$ $CCID_{50}$/ml of an irrelevant adenovirus rAd_SCR^{shRNA} (Vector Biolabs) and rAd_NPPRV1^{shRNA}, respectively (Fig. 18). Forty-eight hours later, the animals were challenged with $0.5x10^{5}$ $CCID_{50}$/goat of the virulent strain of PPRV - Côte d'Ivoire-89 (CI 89). Group-3 was injected with 3 doses of M2b liposome+siRNA NPPRV (12 mg siRNA/dose/day) and similarly challenged 48 hours after first dose administration. Animals were examined daily by a veterinarian for clinical signs and a score according to the severity of signs was accorded. Blood samples for virus detectionwere collected on day 1 to day 14 and the day 20 post-challenge. At the end of experiment, animals were euthanized by injection of barbiturates.

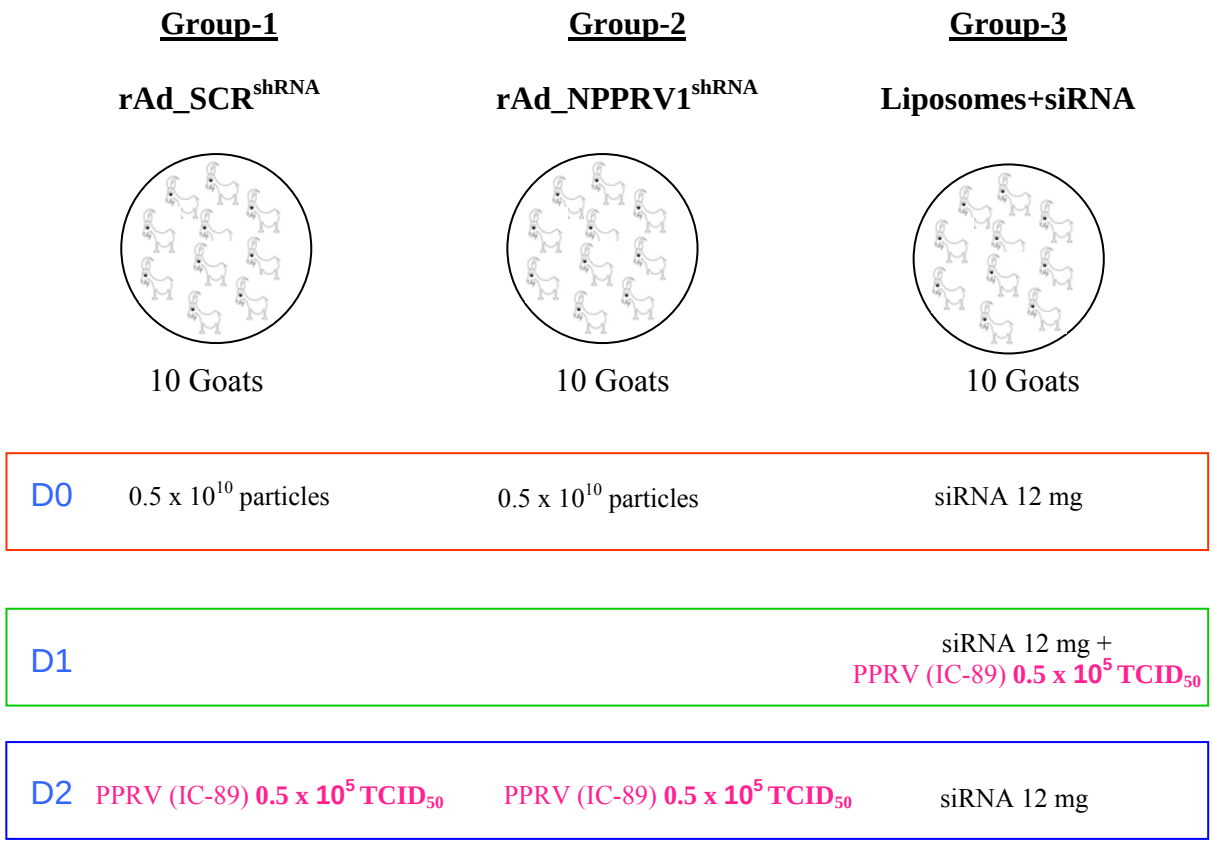


Figure 18: Experimental design for *in vivo* delivery of shRNA/siRNA.

1.2.5.2.1. Assignment of clinical scores

Different clinical scores were assigned according to severity of clinical signs/lesions

- Hyperthermia (equal or greater than 39°C) = 1 point (pt)
- Respiratory difficulties :
    - nasal discharge and lacrimation (mild) = 1 pt
    - nasal discharge and lacrimation (severe) = 2 pts
    - cough= 1 pt
- Disorder of digestive system :
    - mild stomatitis (oral lesions) = 1 pts
    - necrotic stomatitis (oral lesions) = 3 pts
    - diarrhea = 3 pts
- General behavior :
    - good = 0 pt
    - apathy = 1 pt
    - prostration = 2 pts
    - decubitus = 3 pts
- Mortality = 20 pts

1.2.5.2.1.1 Association of scores for ocular and nasal discharges:

Since the mild nasal discharge can be confusing for scoring, more weight was given to scores for ocular discharge than for nasal discharge and similarly if the two signs coexist.

Ocular discharge	Nasal discharge	Combined score
0	0	0
0	1	0
1	0	1
1	1	1
0	2	1
2	0	2
2	1	2
1	2	2
2	2	3

1.3 Results

1.3.1 *In vitro* inhibition of PPRV N protein expression by siRNA NPPRV1 transfected with liposome formulation using various siRNA/lipid mass ratios

Efficacy of siRNA NPPRV1 transfection was measured as % inhibition of nucleoprotein expression and reduction of CPE by PPRV as compared to a positive control which was cell transfected by an irrelevant siRNA and PPRV-infected. Initially, high siRNA/lipid mass ratios (1/90, 1/70, and 1/56) were tested for transfection efficiency in the absence of serum. The transfection time was three hours. The M2b formulation at siRNA/lipid ratio of 1/56 could efficiently transfect siRNA NPPRV1 resulting in an inhibition of PPRV N protein expression up to 64.5% as measured by flow cytometry (Fig. 19). M2b formulation at high siRNA/lipid ratio of 1/56 showed visible toxic effects upon Vero cells. As ultimate objective was to use liposome formulation for siRNA delivery *in vivo*, low toxicity was considered.

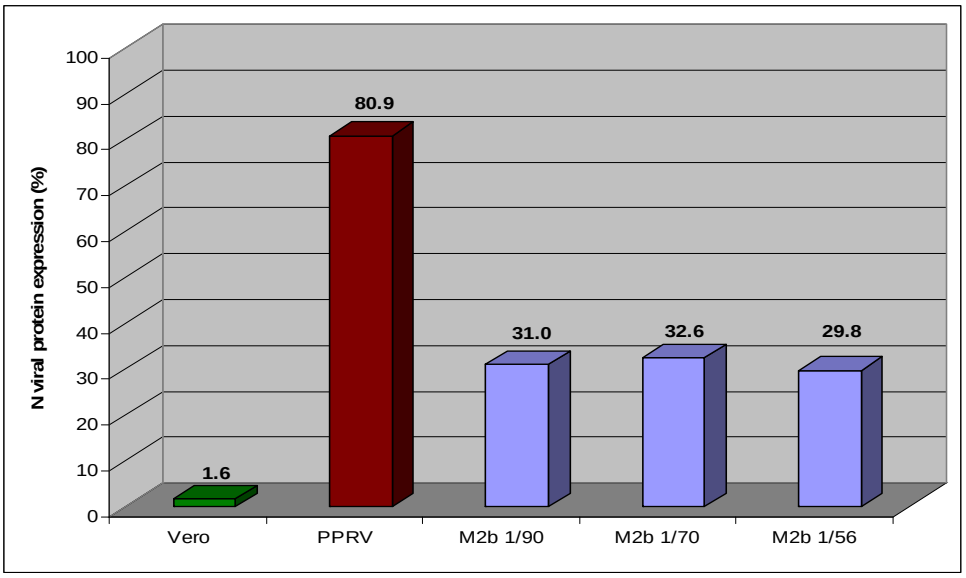


Figure 19: Inhibition of PPRV N protein expression by siRNA NPPRV1 after transfection with liposome formulation M2b.

Therefore, lower siRNA/lipid ratios (1/12.4, 1/10, and 1/6.2) were tested for transfection efficiency of siRNA NPPRV1, both in the absence and in the presence of various concentrations of goat serum. The siRNA/lipid ratio of 1/6.2 was the lowest which could inhibit CPE produced by PPRV to about 80% when transfection was performed in absence of goat serum, while CPE inhibition was about 45% upon transfection in presence of 60% goat serum (Tab. 2 and Fig. 20). Therefore, the liposome formulation M2b with siRNA/lipid ratio of 1/6.2 was selected for *in vivo* delivery of siRNA NPPRV1.

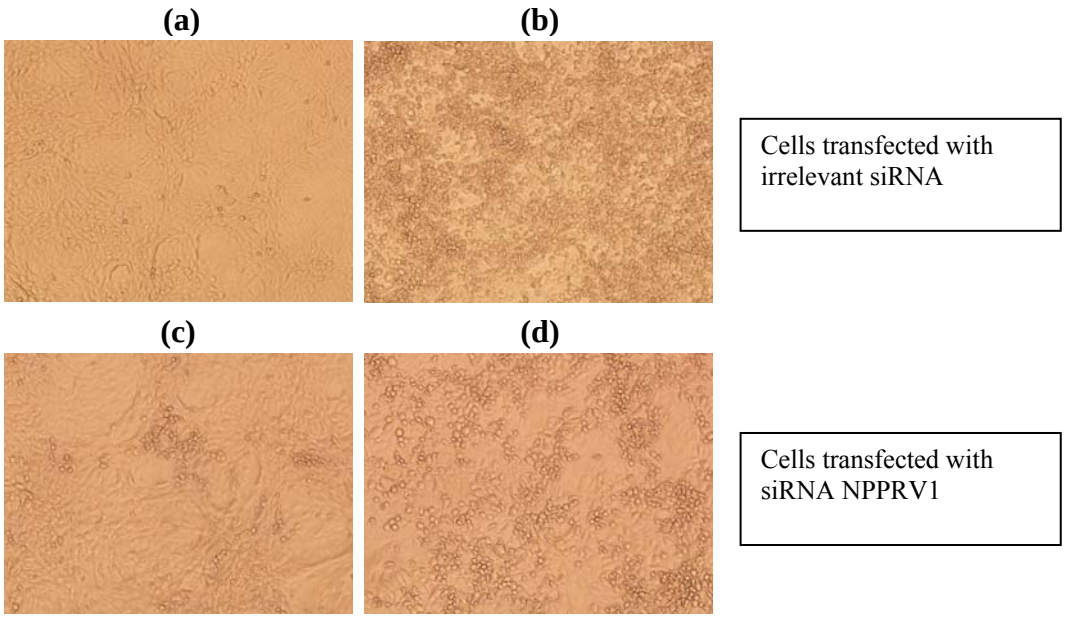


Figure 20: Effect of M2b liposome mediated transfection of siRNA NPPRV1, using siRNA/lipid ratio of 1/6.2 (m/m), upon CPE production by PPRV 96 hours after challenge with MOI 0.1. (a) non-infected Vero cells (b) PPRV infected Vero cells (c) transfection in medium without FBS (d) transfection in presence of 30 goat serum

Table 2: Inhibition of CPE produced by PPRV in Vero cells transfected with siRNA NPPRV1 transfected by M2b liposome formulation using various siRNA/lipid (m/m) ratios.

M2b (DOPE+DOTAP+Cardiolipin) 1/1/0.5	**% Goat serum**	**% Inhibition of CPE**
	10	75
siRNA/M2b 1/12.4	30	50
	60	40
	10	80
siRNA/M2b 1/10	30	50
	60	45
	10	80
siRNA/M2b 1/6.2	30	50
	60	45
Lipofectamine™ 2000	0%	85

1.3.2 *In vivo* delivery of siRNA by M2b liposome formulation and recombinant adenoviruses

1.3.2.1 Clinical scores of animals treated with siRNA+M2b, rAd_NPPRV1^{shRNA} and rAd_SCR^{shRNA}.

The group of animals receiving siRNA in the form of lipoplexes (Group-3), had lower scores for occulo-nasal discharges and stomatitis at day 14 to day 20 post-siRNA/shRNA administration as compared to the positive control group (Group-1) and to the group receiving rAd_NPPRV1^{shRNA} (Fig. 21), but the difference was not statistically significant (Mann–Whitney U test, $p<0.01$). While for diarrhea and body temperatures, scores were in fact higher for both treatment groups (Group-2 and Group-3) as compared to the positive control group (Group-1) (Fig. 21).

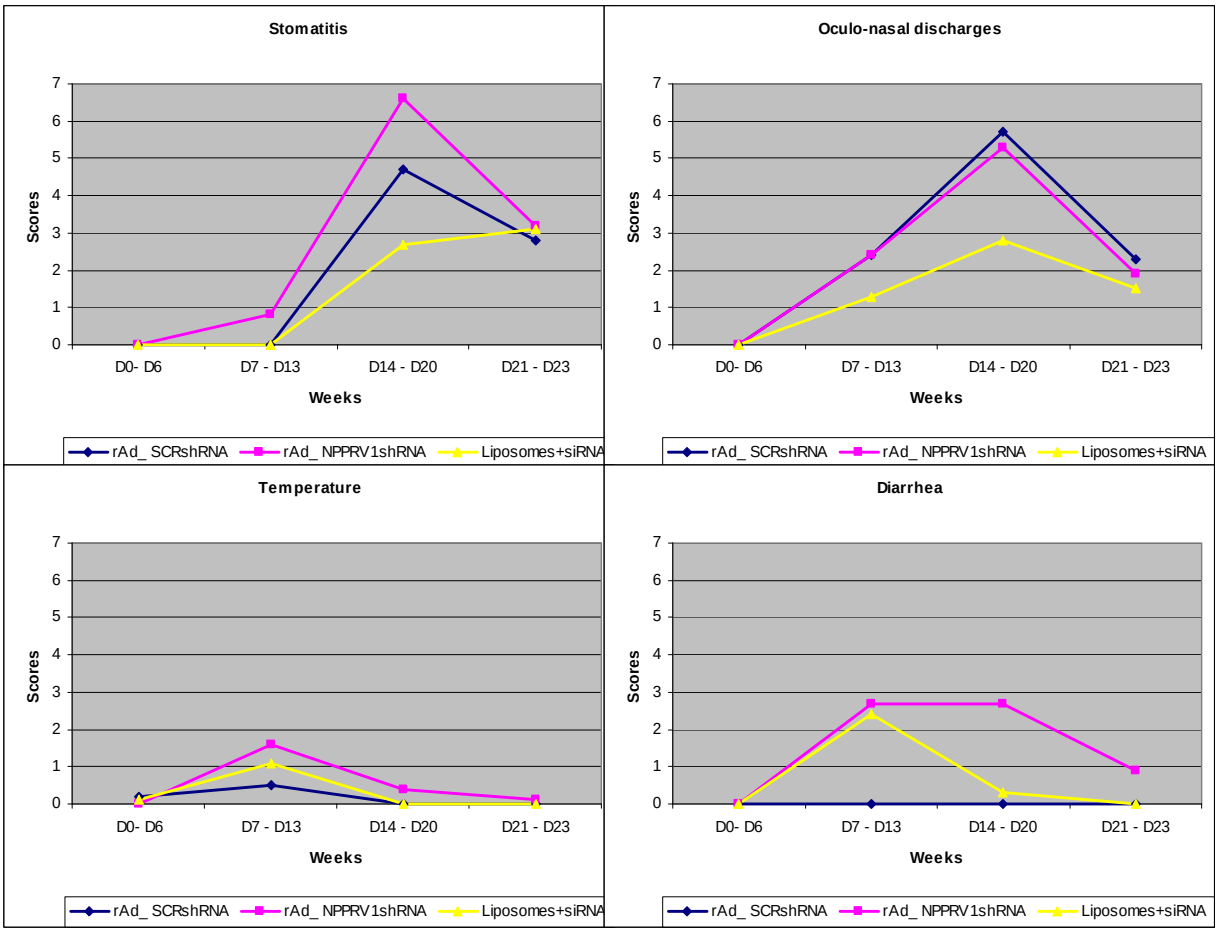


Figure 21: Clinical scores for occulo-nasal discharges, stomatitis, temperature and diarrhea for the group of animals treated with M2b liposome+siRNA NPPRV, rAd_NPPRV1^{shRNA} and rAd_SCR^{shRNA}.

1.4 Discussion

There could be numerous possible reasons for absence of significant therapeutic effect of liposome mediated siRNA delivery and shRNA delivery by the recombinant adenovirus. Firstly, due to problem of precipitation, it was not possible to form lipoplexes using an optimal siRNA/lipid ratio of 1/6.2 and therefore a ratio of 1/2.08 had to be used which is three times lower than expected. This ratio was later tested *in vitro* and it was actually less active and could merely inhibit siRNA mediated inhibition of CPE to about 20 %.

Secondly, the fact that morbilliviruses are primarily lymphotropic and secondarily epitheliotropic [61, 124, 132], highlights the importance to deliver siRNA/shRNA into the lymphatic tissues *in vivo*. The wild-type morbilliviruses use the SLAM or CD150 as a primary receptor [8, 9], which is found on activated T cells, B cells, thymocytes, macrophages and dendritic cells (DCs) [121, 122]. It is reported that measles virus selectively unabled to recognize human SLAM (SLAM-blind) receptors, induced neither clinical signs nor significant viremia, proving that efficient SLAM recognition (on lymphatic tissues) is necessary for viral virulence and pathogenesis [131]. Thus the infection of lymphatic tissues plays a major role in measles virus pathogenesis. However, the lymphatic tissues are not only hard to transfect with liposomes [13] but are also difficult to transduce with adenovirus vectors [354]. Liu *et al*., tested four different liposome based transfectants, including Lipofectamine™ 2000, for siRNA delivery into human T lymphocytes but found no silencing effect [13]. Similarly, in our attempt at transfecting siRNA NPPRV1 with Lipofectamine™ 2000 and subsequent infection of peripheral blood mononuclear cells (PBMCs) of goats with vaccine strain of PPRV, we did not find any inhibitory effect upon NPPRV expression (data not shown). Moreover, it is reported that human adenovirus type 5 could transduce only 4% of human T cells [354]. Similarly we tested transduction of goat PBMCs with human adenovirus type 5 expressing green fluorescent protein (GFP) and found that only 3% of PBMCs could be transduced (data not shown).

There are studies that report successful therapeutic effect of adenoviruses expressing shRNA *in vivo* against foot and mouth disease virus (FMDV) [15, 16]. Chen *et al*., could protect swine from a major clinical disease through adenoviral vectors targeting FMDV, however they could only achieve a limited inhibition of FMDV infection, and they sited the different tissue distribution of rAd5 and FMDV as the reason [23]. The better efficiency of adenoviral vectors against FMDV infection *in vivo* may be due to relative similarity of tropism of the two viruses. Both the adenovirus type 5 and field isolates of FMDV use arginine-glycine-aspartate

(RGD) motifs on their capsids to interact with the $\alpha_v\beta$ subclass of integrins for internalization into host cells, although FMDV uses $\alpha_v\beta_6$ as its primary receptor and it can use $\alpha_v\beta_3$ [355, 356], while adenovirus type 5 uses $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [312]. Finally, the fact that lymphatic tissues are primary sites of replication for morbilliviruses and are very difficult to transduce or transfect transiently as compared to epithelial tissues, can preclude therapeutic implications. Thus from therapeutic point of view, *in vivo* delivery of siRNAs/shRNAs lymphatic tissues, in addition to epithelial tissues, may also be indispensable to control morbillivirus infections.

This experiment *in vivo* showed that better viral and chemical vectors are needed for future *in vivo* delivery and experiments on animals of large size like goats under L3 bio-security, are not only cumbersome, but are also prohibitively expensive. Therefore, a more economical and practical small animal model is needed for initial screening of vectors before their ultimate test in small ruminants.

PART 2:

Potential of adenovirus and baculovirus vectors and cell penetrating peptides (CPPs) for the delivery of shRNA/siRNA against peste des petits ruminants virus.

2.1 Introduction

Therapeutic application of siRNAs requires correct delivery of the molecules into the cell cytoplasm which poses significant problems *in vivo*. Successful delivery of siRNAs/shRNAs by chemical and viral vectors *in vitro* is not always reflected in the form of effective *in vivo* results. Our preliminary attempt at *in vivo* delivery of siRNA/shRNA through liposome and adenovirus vectors did not give promising results (for further information, see Chapter 1 of this document). This entailed us to search for more efficient viral or non-viral vectors which could be possibly used for future siRNA/shRNA delivery *in vivo*.

The adenoviruses were one of the first viral vectors to be used for gene therapy *in vitro* [305] and are the most commonly utilized viral vectors [306]. Replication deficient adenoviruses have been successfully used *in vitro* and also *in vivo* as shRNA delivery vectors against numerous viruses including measles virus [221, 319, 320] . Adenoviruses have advantages over other viral vectors as they can be produced in high titers and can transduce a broad range of cell types [357]. Whereas use of baculoviruses like *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), as shRNA delivery agents have shown promising results *in vitro* against viral infections [332, 333] [334, 335]. Moreover, these recombinant **Bac**ulovirus gene transfer into **Mam**malian cells, known as BacMam baculoviruses, having mammalian cell-active expression cassettes, are capable of transducing a wide variety of cells including non-dividing cells [330] and primary cells [331].

Among the various chemical vectors used for siRNA delivery, cell penetrating peptides (CPPs) are unique since they produce very low or undetectable cytotoxicity [264]. PepFect6 (PF6) and PepFect14 (PF14) are new chemically modified CPPs, developed by team of Ülo Langel (Department of Neurochemistry, Stockholm University, Sweden), which form stable non-covalent complexes with siRNA and are capable of efficient transfection of siRNAs [358] (Fig. 22). Most of siRNA molecules transfected by chemicals, remain trapped in endosomes and are ultimately destroyed, therefore the trafficking of siRNAs from endosomes into the cytoplasm represents a major rate-limiting step for many delivery approaches [359]. The presence of a modified stearyl moiety on both PF6 and PF14 help these peptides to have better interaction with plasma membrane whereas a chloroquine analogue coupled to PepFect6 results in an efficient endosomal escape [358]. Among the two peptides, the PF14 is more serum resistant [33], whereas PF6 can deliver the siRNAs more efficiently into various cell types including lymphocyte suspension cells and primary embryonic stem cells [360]. The PF6 mediated delivery of siRNAs is independent of cell confluence and does not induce

transcriptomic or proteomic changes [360]. Moreover, it is capable of efficient delivery of siRNA *in vivo* in mouse models as well [360].

	Peptide Sequence
PepFect 6	Stearyl-AGYLLGK[$KK_2sa_4qn_4$]INLKALAALAKKIL-NH_2
PepFect 14	Stearyl-LLOOLAAAALOOLL -NH_2

Figure 22: Sequences of Pepfect6 and PepFect14 peptides [358]

In this study, a recombinant replication deficient human adenovirus type 5 and a recombinant baculovirus expressing shRNA (NPPRV1) were compared for their inhibition activity against PPRV replication *in vitro*, referring to the inhibition previously achieved with siRNA delivered by transfecting reagents. Additionally, two novel chemically modified CPPs, the PF6 and PF14 [358], have also been tested for delivery of siRNA NPPRV1 and inhibition of N gene expression by PPRV *in vitro*. These three delivery systems are potential candidates for *in vivo* studies after validation *in vitro*.

2.2 Material and Methods

2.2.1 Cell culture

Vero cells were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% (vol/vol) heat-inactivated bovine calf serum (FBS), 2 mM L-glutamine at 37°C and 5% CO2 in a humidified incubator. Vero cells were used for the production and titration of PPRV and for transduction with the recombinant baculovirus (rBac_NPPRV1^{shRNA}) and the recombinant adenovirus (rAd_ NPPRV1^{shRNA}) vectors. The 293A cells (Invitrogen) were used for the amplification and titration of rAd_NPPRV1^{shRNA}. These cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) FBS at 37°C with 5% CO2. For production and titration of rBac_NPPRV1^{shRNA} and rBac_eGFP^{shRNA}, SF-21 cells were cultured in Grace's insect cell culture medium supplemented with 10% (vol/vol) FBS at 27°C.

2.2.2 Preparation of recombinant viruses

2.2.2.1 Construction of adenovirus rAd_NPPRV1^{shRNA}

The construction of the replication deficient adenovirus vector expressing shRNA NPPRV1 (rAd_ NPPRV1^{shRNA}) is already described in Chapter 1.

2.2.2.2 Construction of the baculovirus rBac_NPPRV1^{shRNA}

Baculovirus vectors expressing shRNA NPPRV1 (rBac_NPPRV1^{shRNA}) and a shRNA against GFP gene (rBac_EGFP^{shRNA}) were constructed and provided to us by Dr. Günther M. Keil (Friedrich-Loeffler-Institut, Germany). Briefly, an insect cell-active GFP-expression cassette was integrated into the commercially available pFastBac Dual to yield plasmid pBacPH_GFPpolyA [361]. This plasmid was used to integrate a blunt ended BsrG1 fragment (400 bp) from pENTR/U6/ NPPRV1^{shRNA} (Fig. 23). The resulting plasmid pBacPH_GFP_NPPRV-1^{shRNA} was used to generate the recombinant baculovirus (rBac_NPPRV1^{shRNA}), as recommended in the Bac-to-Bac® Baculovirus Expression Systems kit (Invitrogen). rBac_NPPRV1^{shRNA} was amplified on SF21 cells. The budded baculovirus from insect cell culture medium was concentrated by ultracentrifugation on sucrose gradient and the viral titers were determined by end-point titration method of Reed and Muench (1938) and expressed as $CCID_{50}$ /ml.

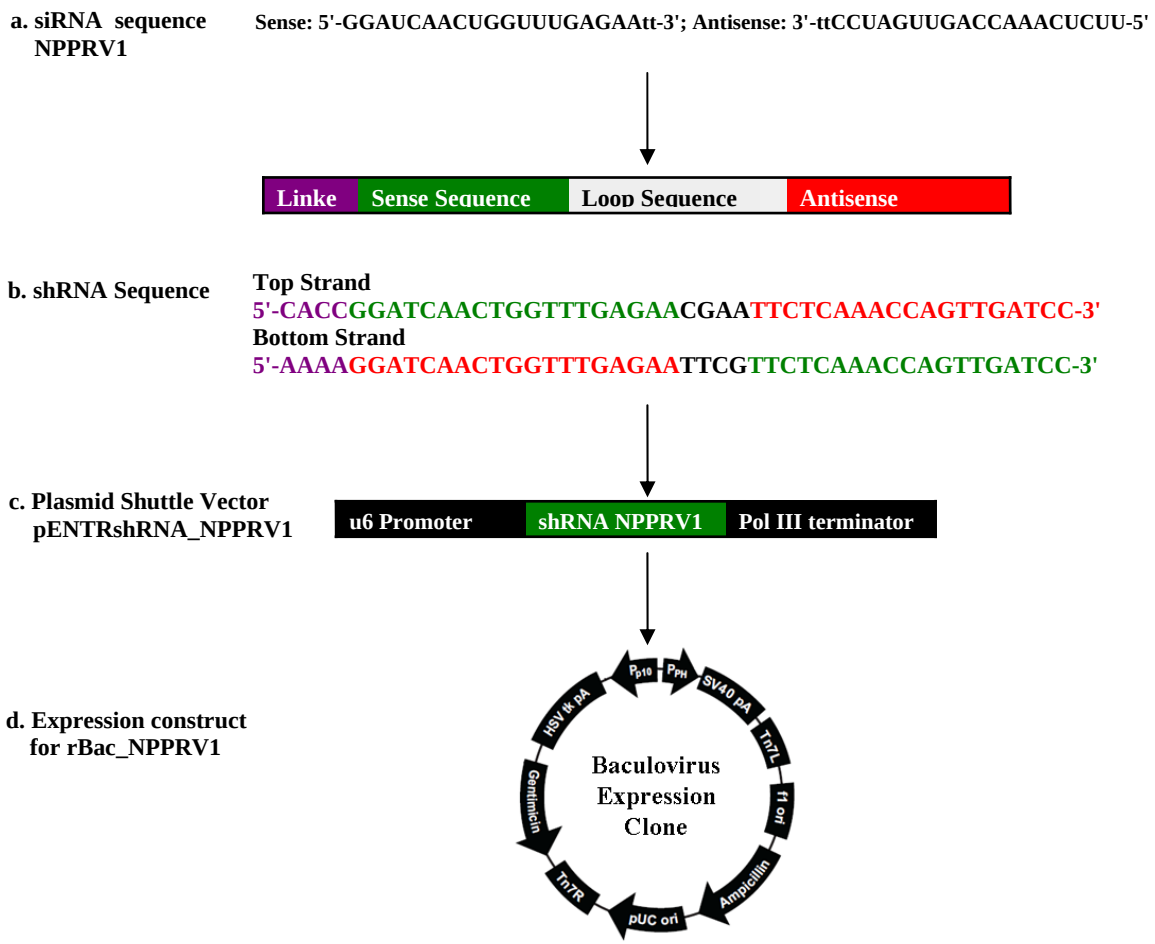


Figure 23: Schematic representation of (a) siRNA NPPRV1 sequence (b) shRNA sequence, (c) plasmid shuttle vector, and (d) expression plasmid for rBac_shRNA_NPPRV1.

2.2.3 Adenoviral and baculoviral transductions and PPRV challenge:

To investigate the interfering activity of shRNA expressed by rBac_NPPRV1^{shRNA} and rAd_NPPRV1^{shRNA}, the Vero cells were first transduced with various transduction doses (MOIs of 25, 50, 100, and 200) and challenged with a low dose of PPRV (MOI of 0.01). Later, the effect of transduction with higher MOIs of the two recombinant viruses (MOIs of 100, 200, 300, and 400) and a challenge with both a low (MOI 0.01) as well as a high (MOI of 0.1) dose of PPRV were assessed.

For transductions with the recombinant adenoviruses and baculoviruses, Vero cells of 48 hours were trypsinized and 10^5 cells were plated in 24 well cell culture plates. Twenty four hours after plating, culture medium was removed. Then, various MOIs of rAd_NPPRV1^{shRNA} and an adenovirus expressing scrambled shRNA, rAd_ SCR^{shRNA} (Vector Biolabs), were added in a final volume of 400 µl per well and the plates were incubated overnight at 37°C, 5% CO2. Next, the inocula were removed and 1 ml of EMEM with 5% of FBS was added to each well. For baculovirus transduction, the culture medium was removed and the cells were washed twice with Dulbecco's phosphate-buffered saline containing calcium and magnesium (Sigma Aldrich). Then, rBac_NPPRV1^{shRNA} and rBac_eGFP^{shRNA} expressing an irrelevant shRNA were added to the wells using various MOIs in a final volume of 300 µl per well. The plates were incubated for 30 min at 27°C under agitation (100 rpm) and then centrifuged at 27°C for 1 hour at 600 g. Inocula were discarded and 1 ml EMEM was added to each well.

Cells were finally challenged with PPRV (MOIs 0.01 or 0.1), 24 and 72 hours post-transduction with the recombinant baculovirus and adenovirus, respectively. To assess the antiviral effect, PPRV cytopathic effects (CPE) were scored from 0 to 100%, at 72 and 96 hours post-infection with PPRV MOI 0.1. In addition, cell supernatants were collected 48, 72 and 96 hours post-infection and titrated using 10 fold serial dilutions according to Reed & Muench [352]. Viral titers were expressed in $CCID_{50}$/ml. The relative expression of PPRV nucleoprotein was also measured 96 hours post-infection by flow cytometry as described by Servan de Almeida *et al*. [353]. Moreover, to confirm that the inhibitory effect upon PPRV replication was due to specific silencing by the two recombinant viruses, Vero cells were transduced and challenged with rinderpest virus (RPV). The two recombinant viruses never showed any inhibitory effect upon CPE produced by RPV (data not shown).

2.2.4 Transfection of siRNA NPPRV1 with PF6 and PF14

The transfection efficacy of PF6 and PF14 was tested on 6 x 10^4 Vero cells grown in 24 well cell culture plates. After 24 hours of growth, medium was removed, cell layer was washed twice with PBS, and 450 µl of DMEM (either without or with variable FBS concentrations) was added to each well. The siRNAs and PepFects were dissolved in RNAse free water. The siRNAs were complexed with PF6 using a molar ratio of 1/10. While for PF14, siRNA/PF14 molar ratio of 1/10 was initially tested and found less efficient upon NPPRV protein expression. Therefore, siRNA/PF14 ratios of 1/5, 1/10, and 1/15 were tested. The complexes were incubated at room temperature for one hour. Next, 50 µl of complexes were added to the duplicate wells. Firstly, final siRNA concentrations of 12.5, 25, 50, and 100 nM /well were transfected with PF6 in the absence of FBS. Later, the siRNA concentration of 100 nM (the most effective concentration) was transfected with PF6 in the presence of various FBS percentages. For transfection with PF14, a final siRNA concentration of 100 nM was used and transfections were performed in the medium with no or variable FBS percentages. Higher siRNA/PF14 ratios and FBS percentages could not be tested owing to limited availability of PF14. Plates were incubated for 4 hours at 37°C with 5% CO2. Next, one ml of EMEM with 5% FBS was added to the wells and plates were further incubated for 20 hours at 37°C with 5% CO2. Finally, the cells were challenged with PPRV at MOI of 0.1 and relative expression of PPRV nucleoprotein was measured by flow cytometry, 96 hours post-challenge. The efficacy of siRNA transfection was measured as % inhibition of nucleoprotein expression by PPRV as compared to an irrelevant siRNA (targeting nucleoprotein gene of RPV). Lipofectamine™ 2000 (Invitrogen) was used as a control of siRNA transfection, according to the supplier's protocol.

2.3 Results

2.3.1 rBac_NPPRV1^{shRNA} and rAd_NPPRV1^{shRNA} challenged with PPRV MOI of 0.01 inhibit PPRV progeny production 96 h post challenge

Both recombinant viruses could inhibit PPRV progeny *in vitro* when transduced cells were challenged with PPRV at MOI of 0.01. However, the rAd_NPPRV1^{shRNA} at MOI of 200 could only reduce PPRV titers to 0.79 $\log_{10}$ with (Fig. 24). In contrast, the rBac_NPPRV1^{shRNA} in the same MOI could inhibit PPRV titers by more than 2 $\log_{10}$ (Fig. 24).

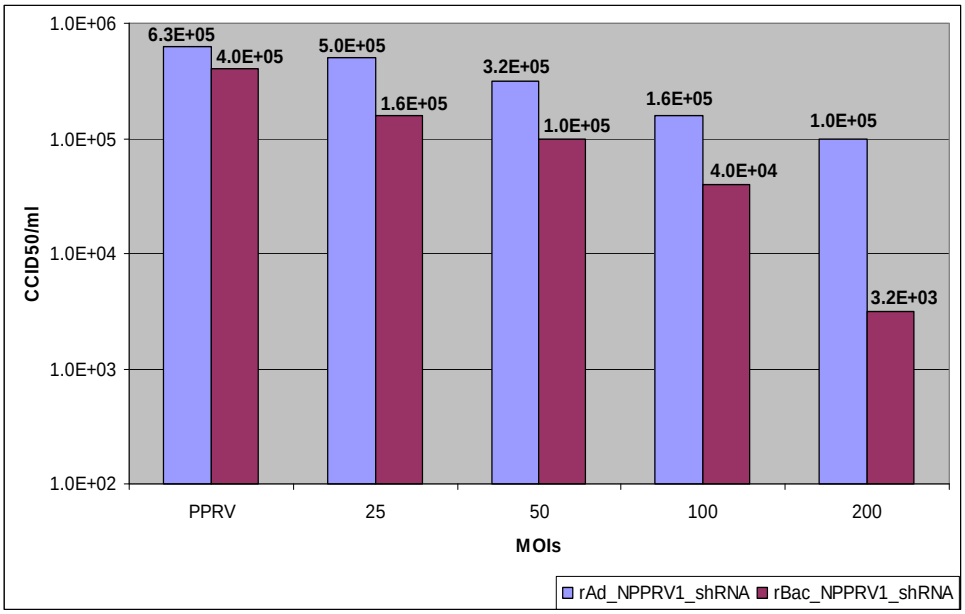


Figure 24: Inhibition of PPRV progeny production by rAd_NPPRV1^{shRNA} and rBac_NPPRV1^{shRNA} 96h post-challenge with PPRV MOI of 0.01.

2.3.2 Effect of higher transduction doses of rBac_NPPRV1^{shRNA} and rAd_NPPRV1^{shRNA} and challenge doses of PPRV, upon inhibition of PPRV progeny production after over time

The effects of transduction with higher MOIs of the two recombinants on a higher challenge dose of PPRV and the time/effect relationship upon PPRV progeny production were assessed. Vero cells were transduced with the two recombinant viruses using MOIs of 100, 200, 300, and 400 and challenged with a low (MOI of 0.01) and high (MOI of 0.1) doses of PPRV.

The rAd_NPPRV1^{shRNA} transduction at MOI of 200, after challenge with PPRV MOI of 0.01 and 0.1, inhibited PPRV progeny titers to 0.5 and 0.7 $\log_{10}$, respectively (Fig. 25). The CPE

reduction rarely exceeded 50% (Tab. 3). Transduction with higher MOIs of rAd_NPPRV1^{shRNA} did not improve the performances of the adenovirus but, in contrast, had a toxic effect on the cell culture.

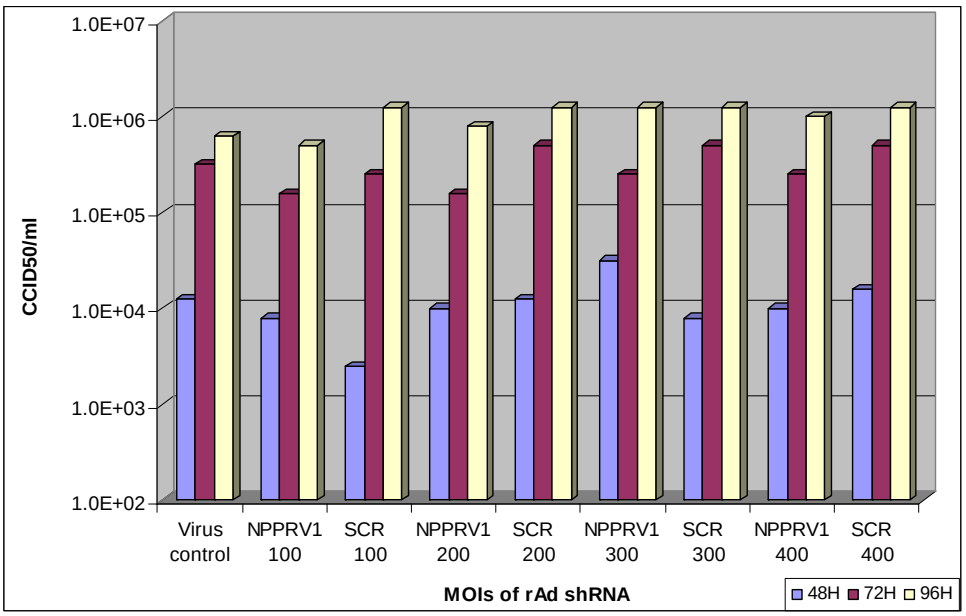
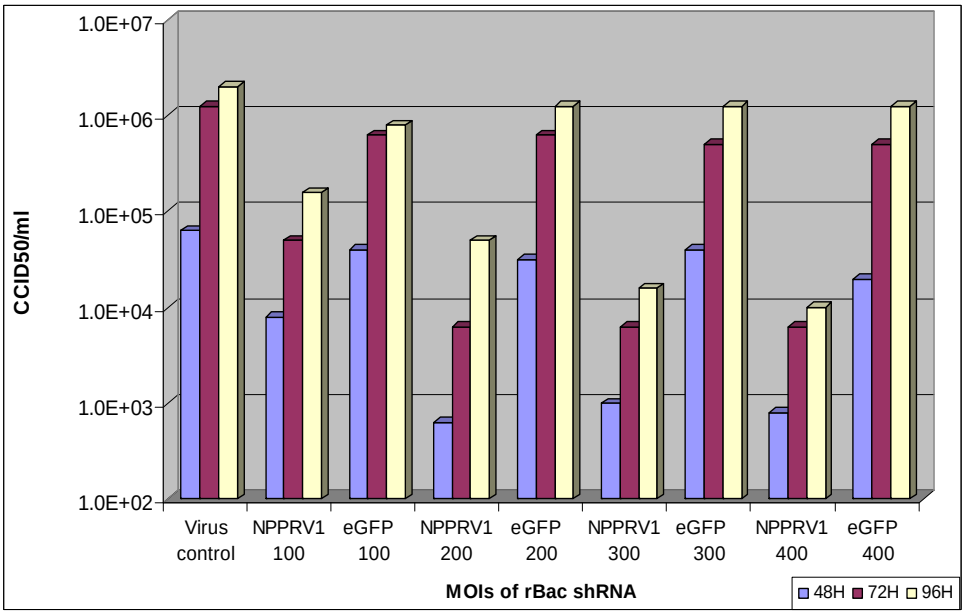


Figure 25: Inhibition of PPRV progeny by rAd_NPPRV1^{shRNA} 48h, 72h and 96h post-infection with PPRV MOIs of 0.1. rAd_NPPRV1^{shRNA} expressed shRNA against NPPRV gene while the rAd_ SCR^{shRNA} expressed a scrambled shRNA and was used as a control.

In contrast, the rBac_NPPRV1^{shRNA}, at MOI of 400, could induce a maximum inhibition of PPRV titers by about 2.1 log_{10}, 96 hours post-challenge with PPRV MOI of 0.01 (Fig. 26a). While upon challenge with PPRV MOI of 0.1, rBac_NPPRV1^{shRNA} transduction at MOI of 400 inhibited the PPRV CPE up to 87.5% and reduced PPRV progeny virus titers by 2.19 log_{10} at 72 and 96 hours post-infection, respectively (Tab. 3 and Fig. 26b). This inhibitory effect on PPRV replication was reflected by an up to 73% reduction of PPRV nucleoprotein expression by rBac_NPPRV1^{shRNA} measured by flow cytometry, 96 hours post-challenge (Fig. 27). No difference in CPE expression by PPRV was found between un-transduced and PPRV infected cells and cells transduced with the two recombinant viruses expressing irrelevant or scrambled shRNA and infected by PPRV.

(a)



(b)

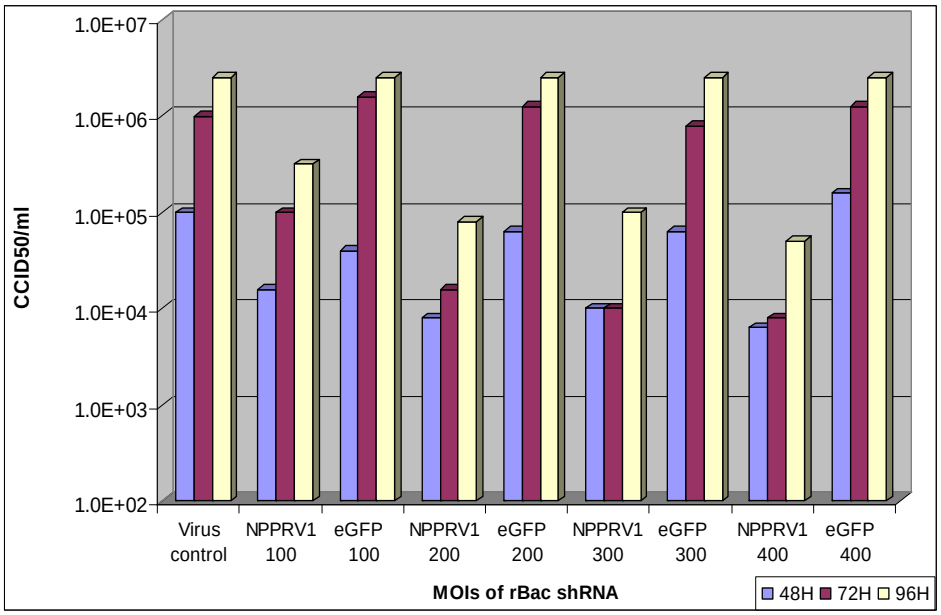


Figure 26: Inhibition of PPRV progeny virus production by rBac_NPPRV1^{shRNA} 48h, 72h and 96h post-infection with PPRV MOIs of (a) 0.01 (b) 0.1. rBac_ eGFP^{shRNA} expressing an irrelevant shRNA was used as control.

Table 3: Effect of rBac_NPPRV1^{shRNA} and rAd_NPPRV1^{shRNA} upon percentage of CPE induced by PPRV at 72h and 96h post infection.

	72h	**96h**
Virus control	75	100
rBac_NPPRV1^{shRNA} MOI 100	25	50
rBac_NPPRV1^{shRNA} MOI 200	25	25
rBac_NPPRV1^{shRNA} MOI 300	25	25
rBac_NPPRV1^{shRNA} MOI 400	12.5	25
Virus control	100	100
rAd _NPPRV1^{shRNA} MOI 100	75	100
rAd _NPPRV1^{shRNA} MOI 200	75	100
rAd _NPPRV1^{shRNA} MOI 300	75	100
rAd_NPPRV1^{shRNA} MOI 400	75	100

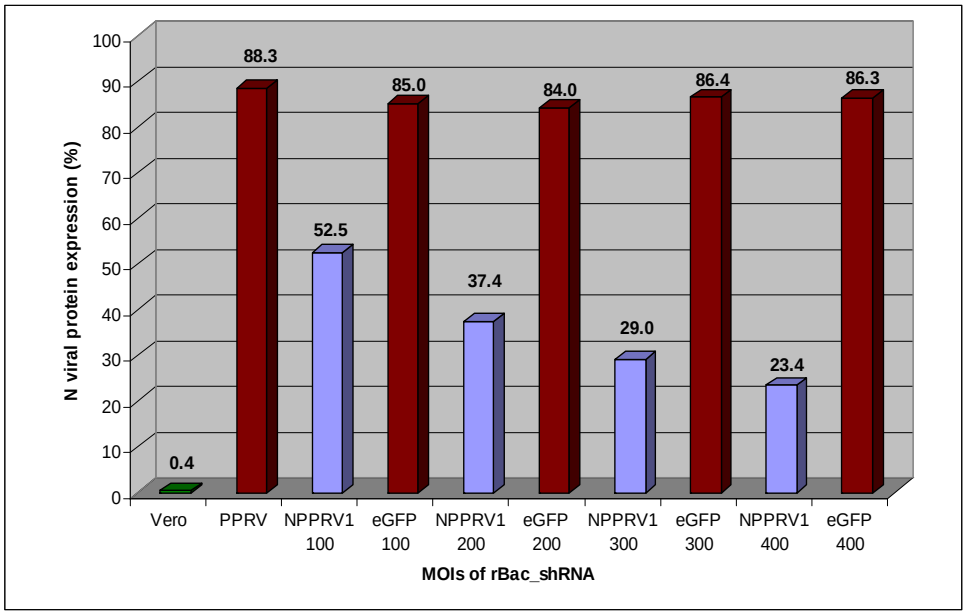


Figure 27: Inhibition of PPRV N protein expression by rBac_NPPRV1^{shRNA} measured by flow cytometry.

2.3.4 siRNA NPPRV1 delivered by PepFect6 and PepFect14 inhibits nucleoprotein expression by PPRV

PF6 is capable of efficiently transfecting siRNA at siRNA/PF6 molar ratio of 1/10. Transfection of siRNA NPPRV1 by PF6 at final concentration of 100 nM inhibits N gene expression by more than 99% while transfection of the irrelevant siRNA NRPV1 has no inhibitory effect (Fig. 28). However, its efficiency declines when transfection is performed in the presence of increasing FBS percentages and no inhibitory effect of siRNA NPPRV1 is

found when transfection is performed in 100% of FBS (Fig. 29). PF14 is apparently less efficient in transfecting siRNAs compared to PF6 at siRNA/PF14 ratio of 1/10 (Fig. 30). However an increase in siRNA/PF14 ratio does increase the inhibitory effect of siRNA (Fig. 31). Moreover, transfection of siRNA with PF14 appears to be less affected by presence FBS.

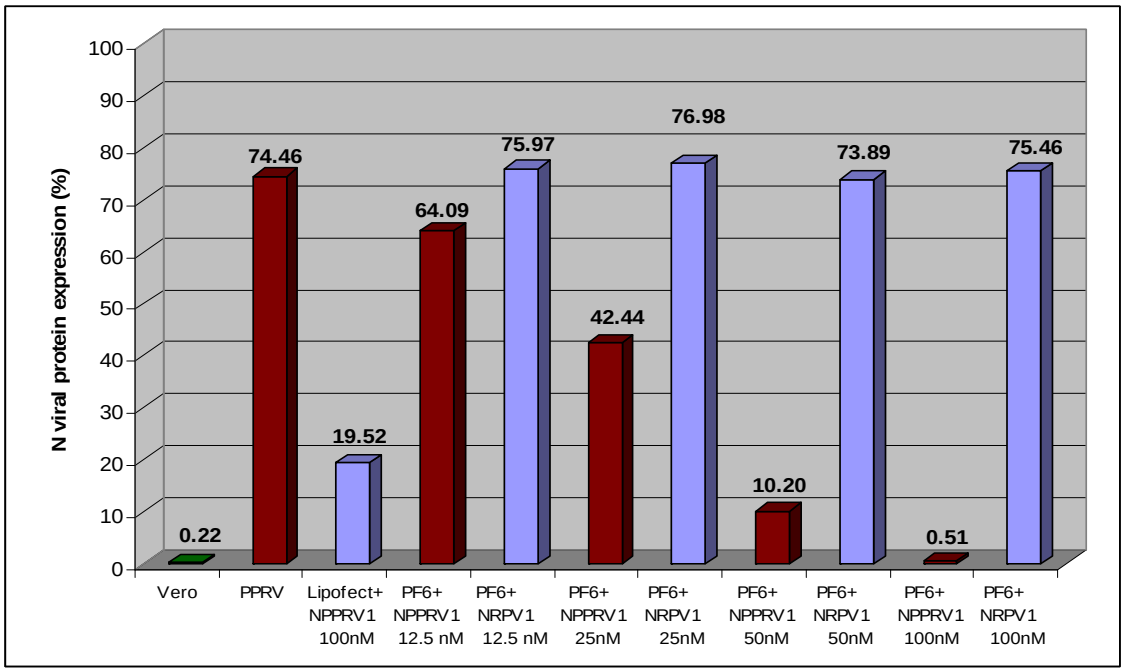


Figure 28: Inhibition of PPRV N protein expression by various siRNA NPPRV1 doses transfected by PF6, measured by flow cytometry.

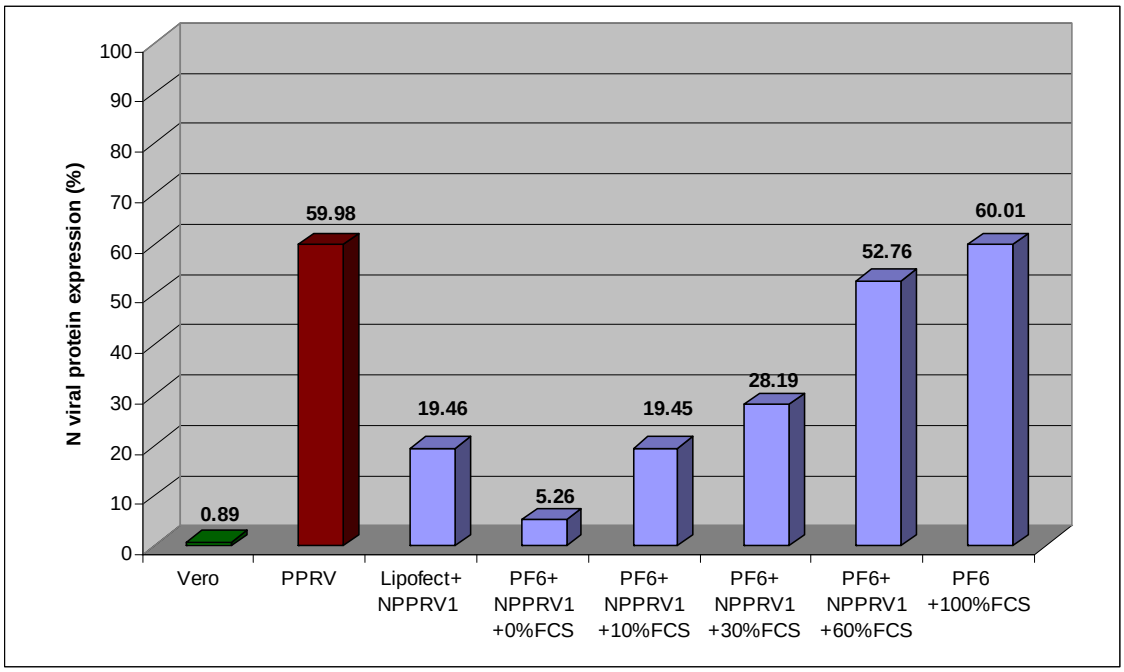


Figure 29: Inhibition of PPRV N protein expression by siRNA NPPRV1 transfected by PF6 upon transfection in the absence or presence of various FBS percentages, measured by flow cytometry.

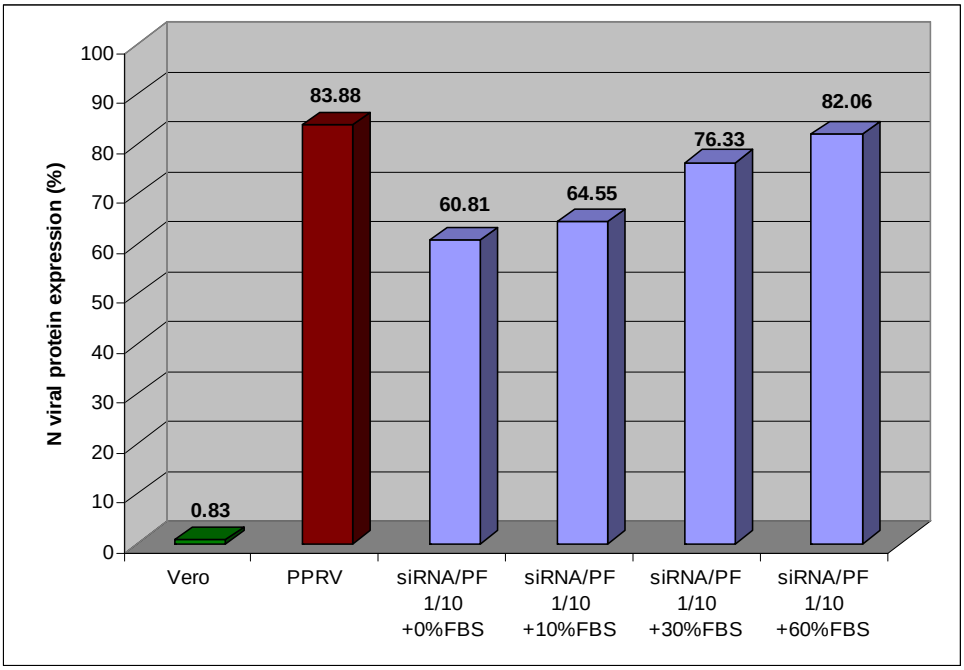


Figure 30: Inhibition of PPRV N protein expression by siRNA NPPRV1 transfected by PF14 upon transfection in the absence or presence of various FBS percentages, measured by flow cytometry.

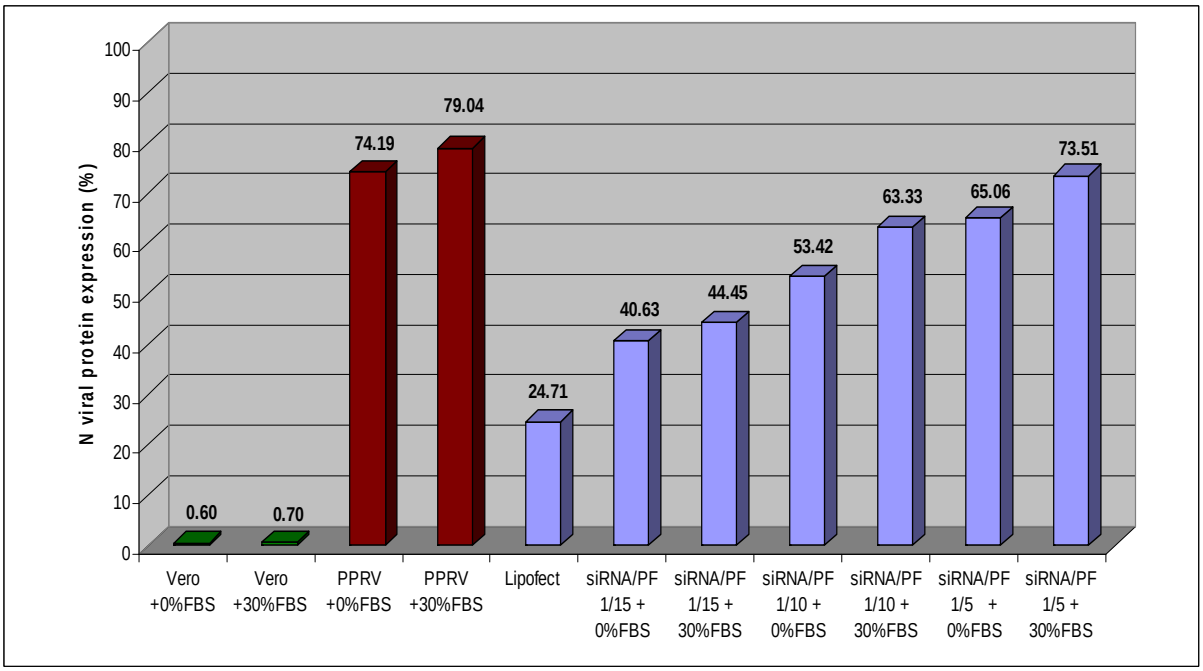


Figure 31: Inhibition of PPRV N protein expression by siRNA NPPRV1 transfected by PF14 in absence or presence of 30% FBS, measured by flow cytometry.

2.4 Discussion

This study provides evidence that the recombinant adenovirus or baculovirus expressing active shRNAs can interfere with the replication of PPRV *in vitro*. However, the baculovirus proved to be more efficient than adenovirus vector when used at same MOIs. The low efficiency of the adenovirus against PPRV replication differs from other studies in which other pathogens were targeted. *In vitro*, shRNA-expressing adenoviruses achieved titer reductions of 3 $\log_{10}$ and 1.39 $\log_{10}$ for porcine picornavirus and circovirus when used at MOIs of 80 and 1000, respectively [225, 362]. Moreover, the results obtained in this study differ also from the results obtained previously by our group using the same recombinant adenovirus in the same conditions (see "State of the art" chapter of this document). The reason for this efficacy discrepancy is not really known. The initial experiments were performed with the rAd_NPPRV1^{shRNA} vector which was produced at CIRAD while later *in vitro* as well as *in vivo* experiments were performed using the rAd_NPPRV1^{shRNA} vector which was produced and purified by another laboratory (using bio-fermentor growth and purification by chromatography). The process may have modified the virus. Moreover it is known that many chromatographic elution buffers used for Adenovirus purification procedures are not suitable for *in vivo* manipulations [363]. Thus the two lots of rAd_NPPRV1^{shRNA} were not the same and it could be at the origin of this discrepancy. Anyhow, it remains to be evaluated. Here, the rAd_NPPRV1^{shRNA} did not reduce PPRV titers more than 0.7 $\log_{10}$ (Fig. 25) and higher concentrations of this vector showed to be deleterious to transduced cells. Whereas, the rBac_NPPRV1^{shRNA} tested in this study, when used at MOI of 400, could reduce PPRV replication by 2.19 $\log_{10}$ (Fig. 26b), which is higher than other comparable studies using baculoviral vector for delivery of shRNA against other viral pathogens *in vitro*. Lu *et al.,* [332] using baculoviral vector expressing shRNA against nucleoprotein of porcine arterivirus, at MOI of 625, could inhibit viral titers up to 0.6 $\log_{10}$. While Suzuki *et al*., [335, 364], reported a decrease in titer for human influenza viruses to a maximum of 0.9 $\log_{10}$, after *in vitro* transduction with baculoviral vector expessing bifunctional shRNA at MOI of 100.

Inhibition of PPRV titer and N viral protein expression by 2.1 $\log_{10}$ and 73% respectively, by rBac_NPPRV1^{shRNA} is lower than the N protein inhibition by 90% and a reduction of PPRV progeny by 3-4 $\log_{10}$ that our group have obtained by siRNA NPPRV1 delivered by Lipofectamine 2000™ [350]. Moreover, highest inhibition of PPRV progeny titers was obtained 72h post-challenge for the higher challenge dose of PPRV (MOI 0.1) but the inhibitory effect tends to decline by 96h (Fig. 26b). This result is surprising since as far as

longevity of action is concerned shRNA expressing viral vectors normally have advantages over chemically synthesized siRNAs. It is reported that less than 1% of the introduced siRNA duplexes remain in the cell 48 hours after administration, whereas the shRNA can be continuously synthesized by the vector in the host cells, therefore, its effect should be much more durable [185]. However, as the viral vectors expressing shRNA used here are replication deficient, shRNA expression is limited and can get diluted over time due to continuous cell division, while the target virus may continue to replicate in non-transduced cells producing ever more mRNA and can also re-infect other cells. Maximum inhibition was observed 96 hours post challenge, when the cells transduced cells with high baculovirus MOIs were challenged with the low PPRV challenge dose (MOI 0.01) (Fig. 26a).

Anyhow, the *in vivo* application of this viral vector can be advantageous when compared to liposomes since baculoviruses do not have deleterious effects on mammalian cells even when used at very high concentrations [339]. Indeed, we did not find any adverse effects of rBac_NPPRV1^{shRNA} in Vero cells even when used at an MOI of 400. On the other hand, baculoviruses seem to be susceptible to complement inactivation [336] that could make it impracticable to *in vivo* application. However, chemical or genetic modification, can overcome this problem [337, 338]. Furthermore, morbillivirus infections occasionally lead to infection of nervous system as well [16, 17], while the baculoviral vectors are not only capable of transducing nervous tissues in mouse upon being injected into brain but have also been reported to cross blood brain barrier [340].

Finally, two new chemical vectors, which are CPPs named the PF6 and PF14, were tested for their ability to transfect the siRNA NPPRV1 in Vero cells. The siRNA NPPRV1 has already been validated to be effective against PPRV at concentration of 100 nM when transfected with Lipofectamine 2000™ [350]. Transfection of siRNA NPPRV1 at 100 nM with PF6 could silence viral protein expression by 99% (Fig. 28) which is higher than around 90% inhibition obtained with Lipofectamine 2000™ [350]. Interestingly, even at 50 nM, siRNA NPPRV1 transfected with PF6 could inhibit protein expression by 86.5% while it was only 78% with Lipofectamine 2000™ [350]. Therefore, PF6 has a better efficacy compared to Lipofectamine 2000™.

The PF14 is less efficient as reflected by a lower inhibition of N gene expression by PPRV (Fig. 30). However a higher siRNA/PF14 ratio of 1/15 had better effects, thus suggesting that a further increase of siRNA/PF14 ratio may improve antiviral activity (Fig. 31). Moreover,

PF14 is less affected in the presence of serum compared to PF6 (Fig. 31 and Fig. 29). However, at the siRNA/PF ratio of 1/10, the PF6 is still around 2.6 and 5.5 times more efficient, when transfection is performed in the presence of 30% and 60% of FBS, respectively. The inhibitory effect of higher concentration of serum in the PF6 and PF14 activities remains to be evaluated.

In conclusion, a recombinant replication deficient adenovirus and a baculovirus expressing shRNA against nucleoprotein of PPRV were constructed and tested *in vitro*. This study shows that both recombinants can inhibit PPRV replication *in vitro*. However, the baculoviral vector was more efficient. A CPP PF6 can deliver siRNA NPPRV1 effectively *in vitro* resulting in an almost complete inhibition of N gene expression by PPRV. PF14, although has a lower transfection efficiency *in vitro*, appears to be more serum resistant.

The PF6 and rBac_NPPRV1^{shRNA} vectors were efficient *in vitro* however whether these would prove equally efficient for inhibition of PPRV replication also *in vivo* needs to be elucidated.

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PART. 3:

First contributions towards the development of a small animal model for the assessment of siRNA activity *in vivo*

3.1 Introduction

Final potential beneficiaries of an antiviral therapy based on siRNA against morbilliviruses are large species like ruminants, dogs and possibly humans. However, the demonstration of *in vivo* efficacy can hardly be established directly in the target species for ethical, safety and economic reasons. Therefore, a small animal model is highly desirable to generate the proof of concept and also demonstrate the safety and efficacy of such an approach. Validated small animal models for morbillivirus infection are not frequent. This is probably due to a long virus-host co-evolution, limiting cross-species transmission.

However, for measles virus, a mouse model for experimental challenge was developed recently [365]. Since we have identified active siRNA against this virus, the use of this model was initially considered. Nevertheless, for intellectual property reasons, this model could not be transferred to our laboratory. Another small animal model based on Marmosets could be used as these have been shown to be susceptible to measles [366, 367]. In addition, the model requires the manipulation of virulent measles virus strains. Although zoonotic diseases are currently investigated in our laboratory, we are not primarily in a human health research unit. The permission to use virulent measles virus strains is therefore a long and tricky process. A lapinized strain of rinderpest virus was shown to induce disease in rabbits [368]. However, the use of rinderpest virus strains is not recommended since this disease is about to be eradicated globally and the propagation of the virus in target or non target species always increases the risk for pathogen escape. In addition, the *in vivo* validation of siRNA against an eradicated disease would have a limited impact.

For PPRV, the third virus for which we have demonstrated an *in vitro* activity of siRNA, no animal model is available. For this reason, we decided to launch a strategy for developing a mouse model for PPRV infection. Recently, mice that are knocked out for the interferon type I receptor (IFNAR-/-) were shown to be highly susceptible to bluetongue virus [369]. The research team that established this model was contacted and collaboration was initiated to test a virulent PPRV challenge on these IFNAR-/- mice. However, these mice never developed disease after PPRV challenge or sero-converted against the virus (data not shown).

An alternative strategy was then designed to enable an *in vivo* assessment of systemic siRNA delivery for efficacious knock down of a morbillivirus gene.

3.2 Development of a non-infectious model to test *in vivo* the delivery of siRNA

In a first approach, a non-infectious model was designed with the objective of generating kinetic and quantitative measurements of RNA interfering activity in a peripheral tissue. This model consisted of the expression of a luminescent Firefly reporter gene downstream of the target sequence of a siRNA directed against nucleoprotein gene of PPRV (named NPPRV1). The expression of this reporter gene is self-contained in the tibialis anterior (TA) muscle of mice after intramuscular injection of the plasmid containing the expression cassette driven by the human cytomegalovirus promoter (pCMV). For the kinetic and quantitative measurement of luminescence, we selected a method based on bioluminescence imaging (BLI) of mice allowing a measurement once a day over a week for all treated animals. Bioluminescence is a chemo-luminescence reaction only achievable by a living organism and produces light that can be imaged by non-invasive techniques. It has been used to monitor specific cellular or genetic activities in the animals. This system is therefore perfectly adapted to test different delivery systems for our siRNA, with a dynamic approach in live animals. In this method, a cooled charged couple detector (CCD) camera is used to quantify photons emitted from a light source localized in deep tissues. Generally, luminescence is preferred for that purpose because there is no such light emission in natural tissues and therefore the signal/noise ratio is very high.

The most often used luminescence marker is the Firefly luciferase which cleaves the luciferin substrate and generates photons that are detected by the camera. We decided to use this reporter gene in our approach. However, the normalization of the signal required later on the use of a second “house-keeping” marker to minimize variability among mice.

3.2.1 Material and methods

3.2.1.1 Production and *in vitro* validation of a siRNA-NPPR1-Firefly_luciferase-2 reporter system

The reporter system was constructed according to the strategy depicted in figure 32. Briefly, the Firefly luciferase-2 (*luc2*) gene was amplified from a commercial reporter plasmid pGL4.51[*luc2*/CMV/Neo] Vector (Promega) using Phusion™ High-Fidelity DNA polymerase (FINNZYMES, Finland) with the following primers: forward sequence 5'-G**GA-TCA-ACT-GGT-TTG-AGA-A**AT-GGA-AGA-TGC-CAA-AAA-CAT-TAA-GAA-GGG-C-3', reverse sequence 5'-TTA-CAC-GGC-GAT-CTT-GCC-GCC-CTT-C-3'. The forward primer consisted in the target sequence of the siRNA-NPPRV1 (in bold), followed by the complementary sequence of the Firefly luciferase-2 gene. This modified gene was then inserted into an expression plasmid vector (pCI-neo Mammalian Expression Vector, Promega) by blunt ended ligation. The expression capacity of this construction was then assessed *in vitro* by transfection of Vero cells with 100 ng of the plasmid and Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's protocol. A positive control of expression consisted of the original commercial plasmid having the *luc2* gene (pGL4.51, Promega). The luciferase gene expression was measured at 24-48 hours post-transfection by ONE-Glo™ Luciferase Assay System (Promega) with a luminometer (VictorII, Berthold-Wallac). Results were expressed as relative luminescent units (RLU). The down-regulation of the siRNA-NPPRV1-Firefly luciferase-2 construction, later on named psiRNA-Fluc, by siRNA NPPRV1 was then validated *in vitro* by co-transfection of both molecules in Vero cells with Lipofectamine™ 2000 (Invitrogen)

3.2.1.2 *In vivo* validation of Firefly luciferase reporter systems

In order to test the *in vivo* delivery of siRNA against the psiRNA-Fluc, a model consisting in a dynamic monitoring of the luciferase expression in mice by bioluminescence imaging (BLI) was developed. The siRNA-Fluc gene in pCIneo plasmid (psiRNA-Fluc) was appropriately diluted in PBS as indicated in the results section and injected under a volume of 60 µl in the TA muscle of mice as previously reported by others [370]. A positive control, consisting of the commercial Firefly luciferase gene under the same pCMV promoter (pGL4.51, Promega), was also used at the optimal dose of 10 µg/60 µl as previously defined (results not shown).

Expression of the reporter gene was measured 48 hours after injection. Measurement was done between 8 to 15 minutes after two separate intra-peritoneal injections of 100 µl of D-

Luciferin potassium salt (150 mg/kg, Caliper Life Sciences) using an IVIS-Lumina (Caliper Life Sciences). For imaging, anesthetized animals were placed in the light-tight chamber of the IVIS-Lumina. Photons emitted from luciferase-expressing plasmids were collected and integrated over three-minute periods. Pseudocolor images indicating photon counts were overlaid on photographs of the mice using the Living Image software version 4.0 (Caliper Life Sciences). Regions of interest (ROIs) were selected manually over the signal intensity.

3.2.1.3 *In vivo* assessment of siRNA delivery against siRNA-Fluc

Ten mice were split into two groups of five. One group received over three consecutive days, the siRNA-NPPRV1 at 40 µg/dose/mouse in a liposome-based emulsion (Aonys, Medesis Pharma, Montpellier) administrated by the intra-rectal route. This delivery system has been previously validated for siRNA delivery in mice targeting other genes (unpublished results). The second group of 5 mice received an irrelevant siRNA (MPPRV10) targeting the matrix protein of PPRV. Twenty four hours after the first administration of the siRNA-NPPRV1-Aonys, all mice received in the left leg 10 µg/60 µl of pGL4.51 and 30 µg/60 µl of psiRNA-Fluc (dose chosen after the previous *in vivo* test). All mice were imaged as previously described, 48 hours after plasmid injection (72 hours after first administration of the siRNA).

3.2.1.4 Inclusion of a second reporter gene to normalize the *in vivo* psiRNA-Fluc

The initial *in vivo* test of siRNA delivery showed that the signal intensity between mice was highly variable and thus required further normalization. The observed variability was ascribed to the lack of accuracy of pDNA injection/expression in the mouse TA muscle. It was decided to develop a protocol based on a co-injection of the Firefly luciferase reporter gene with another reporter gene retaining a stable expression over time. This stable reporter gene would then be considered as a sort of house-keeping gene and serve for normalization.

The Renilla luciferase gene was selected as a second luminescent reporter gene. The enzyme expressed by this gene has a specific substrate that does not cross react with that of the Firefly luciferase gene. Conversely, the D-Luciferine is not used by Renilla. However, the emission spectra of the two substrates are overlapping, thus requiring a sequential injection of the substrates in mice. The plasmid vector pGL4.75[*hRluc*/CM*V*] expressing Renilla luciferase gene under the pCMV promoter was bought from a commercial company (Promega). After *in vitro* validation using the same approach than for the Firefly luciferase gene, the Renilla was probed *in vivo*. Three mice received in both tibialis muscles a co-injection of pGL4.51 (Firefly luciferase, 10 µg) and pGL4.75[*hRluc*/CM*V*] Vector (Promega) (Renilla luciferase,

100 µg) in 60 µl of PBS. Seventy two hours later, the mice were imaged 4 minutes after intravenous injection of Coelenterazine h (the substrate of Renilla, 22 µg in 150 µl). Twenty minutes later, the mice were injected with D-Luciferin as previously described and again imaged.

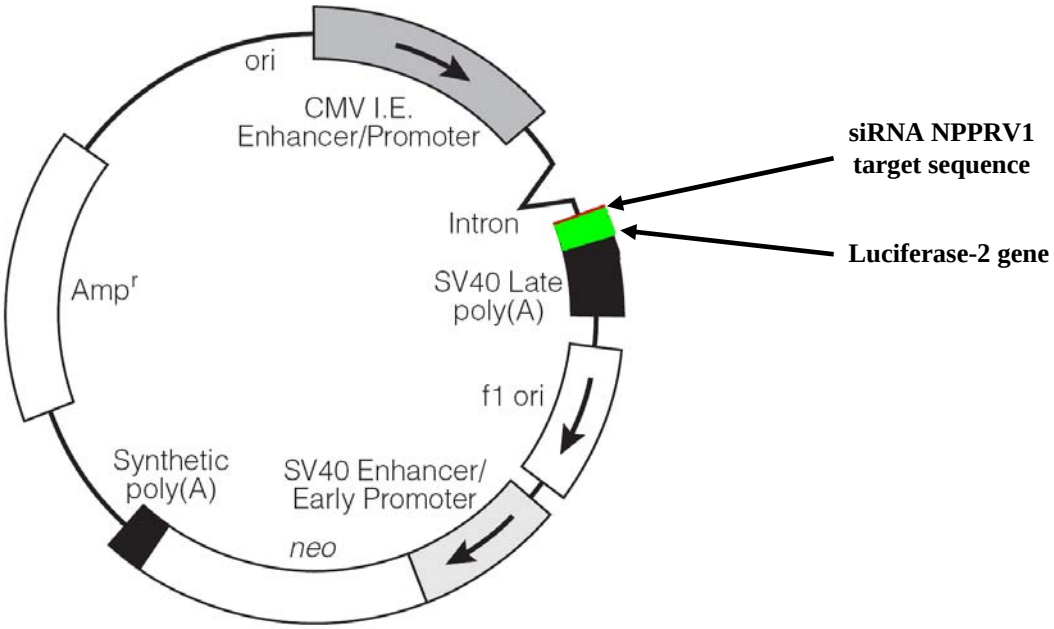


Figure 32: Schematic presentation of siRNA-NPPR1-Firefly luciferase-2 reporter gene "psiRNA-Fluc" construct.

3.2.2 Results

The psiRNA-Fluc construction was achieved and tested *in vitro* in comparison with the commercial Firefly luciferase gene (pGL4.51, Promega). Both constructions have an expression driven by the pCMV promoter. The luciferase expression by psiRNA-Fluc construction was first found to be around 20 times more than the expression of pGL4.51 (data not shown). In a second step, the psiRNA-Fluc was probed in presence of the siRNA-NPPRV1 or an irrelevant siRNA in comparison with PGL4.51. The table 4 shows that siRNA-NPPRV1 specifically inhibited the expression of luciferase by psiRNA-Fluc and not by the original pGL4.51 Firefly reporter. In addition, the irrelevant siRNA could not down-regulate any construction, thus illustrating that the sequence placed upstream of the Firefly luciferase reporter gene was efficaciously targeted by our siRNA.

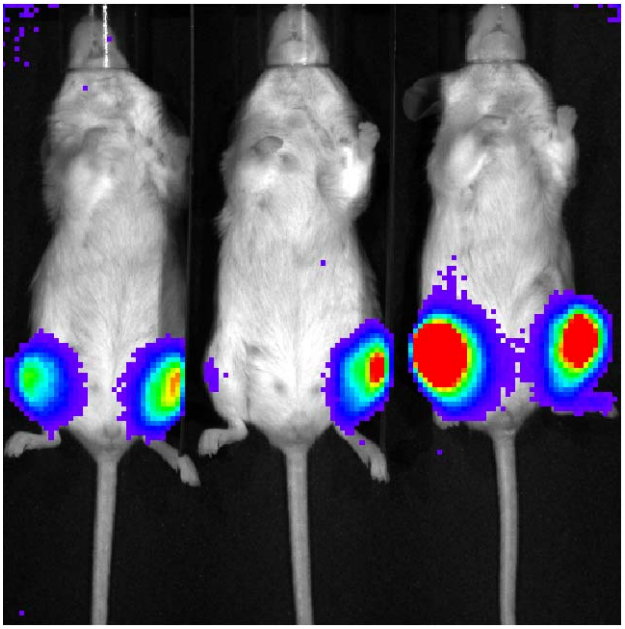
Table 4: *In vitro* inhibition of luciferase expression by psiRNA-Fluc after co-transfection with siRNA NPPRV1.

	pGL4.51 (100 ng)		psiRNA-Fluc (100 ng)		Negative control	
	24h	**48h**	**24h**	**48h**	**24h**	**48h**
20 pmole siRNA NPPRV1	16924	3183	13672	2584	134	174
20 pmole -siRNA IR*	26527	7440	634592	92659		
40 pmole siRNA NPPRV1	17954	6166	9592	2344		
40 pmole-siRNA IR	21335	3047	257299	72207		

*IR: irrelevant

Before testing the delivery of siRNA NPPRV1 *in vivo*, we tried first to calibrate the amount of psiRNA-Fluc plasmid to be injected in the TA muscle. Forty eight hours post injection of the reporter plasmids, the mice were injected with D-Luciferin and imaged. Results are shown in figure 33. The reproducibility of the positive control was not perfect in this assay since the second mouse showed almost no signal. However, the dose/effect of psiRNA-Fluc was considered as acceptable. Since, the production of micrograms of plasmid is expensive and time-consuming, we considered from this trial that a dose of 30 µg/60 µl for psiRNA-Fluc would be satisfactory enough for the next studies although the signal at this dose may be lower than the one resulting from 10 µg/60 µl of pGL4.51 (see mouse 3 in Fig. 33).

(a)



(b)

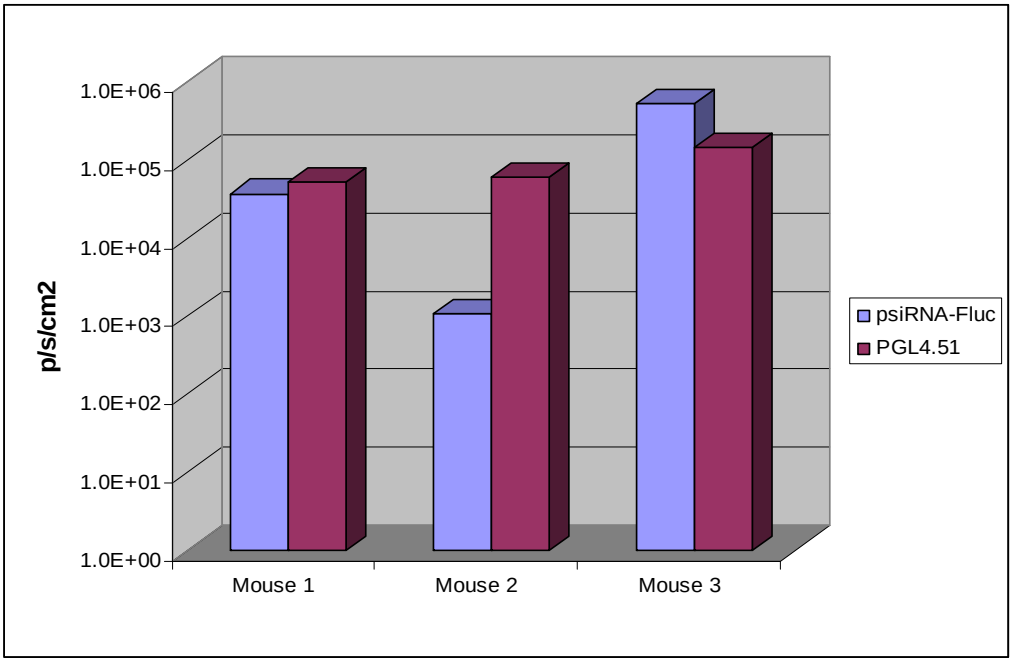
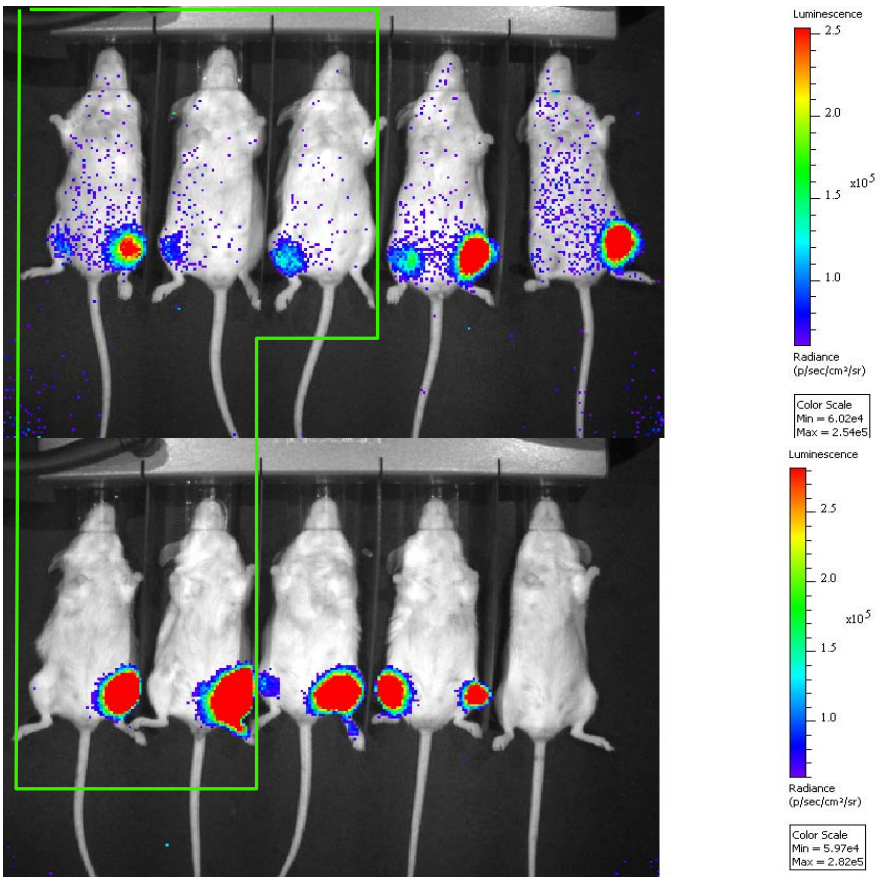


Figure 33: Determination of the optimal dose for psiRNA-Fluc *in vivo*. (a) In the three mice, PGL4.51, considered as the positive control, was injected at a constant dose (10 µg/60 µl) in the left leg. As depicted from left to right, the psiRNA-Fluc was injected in the right leg at 3, 10 and 30 µg/ 60 µl. (b) The histogram shows the total photons measured at each ROI per second and surface (p/s/cm²).

The RNA interference against our psiRNA-Fluc reporter system was tested using a liposome-based formulation developed by a private company (Medesis Pharma, Montpellier). The delivery of siRNA with this system requires three consecutive deposits in contact with the rectal mucosa. Results are shown in Figure 34. For all mice, whatsoever the treatment received, luciferase expression increased from day 1 to day 2 after the plasmid injection. Afterwards, the increase became inconsistent. The expression of pGL4.51 was comparable between the group treated with the specific siRNA-NPPRV1 and the group treated with the irrelevant siRNA. A difference was observed between the two groups when considering the expression of psiRNA-Fluc. However, this difference was not significant because of a high heterogeneity within each group with some non–responding individuals (Fig. 34). This observation confirmed what was already observed in the previous test (Fig. 33). This variability resulted from the quality of plasmid injection and not from the substrate bioavailability after intraperitoneal injection as it persisted over days and after repeated substrate injections. Therefore, we decided to develop a double labeled system to assess the efficacy of plasmid injections and to normalize the test signals.

(a)



(b)

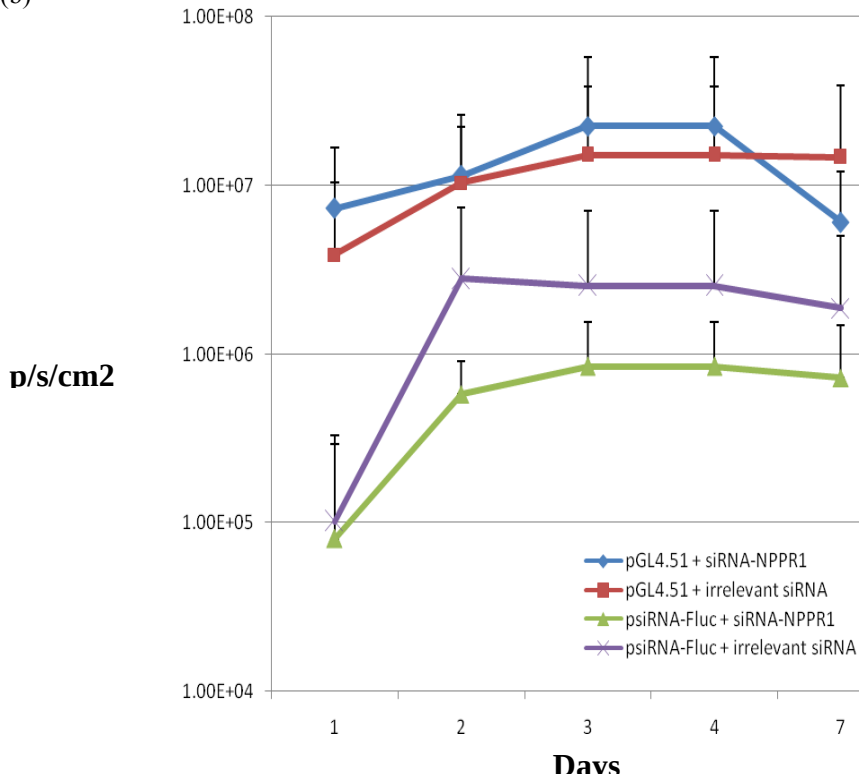
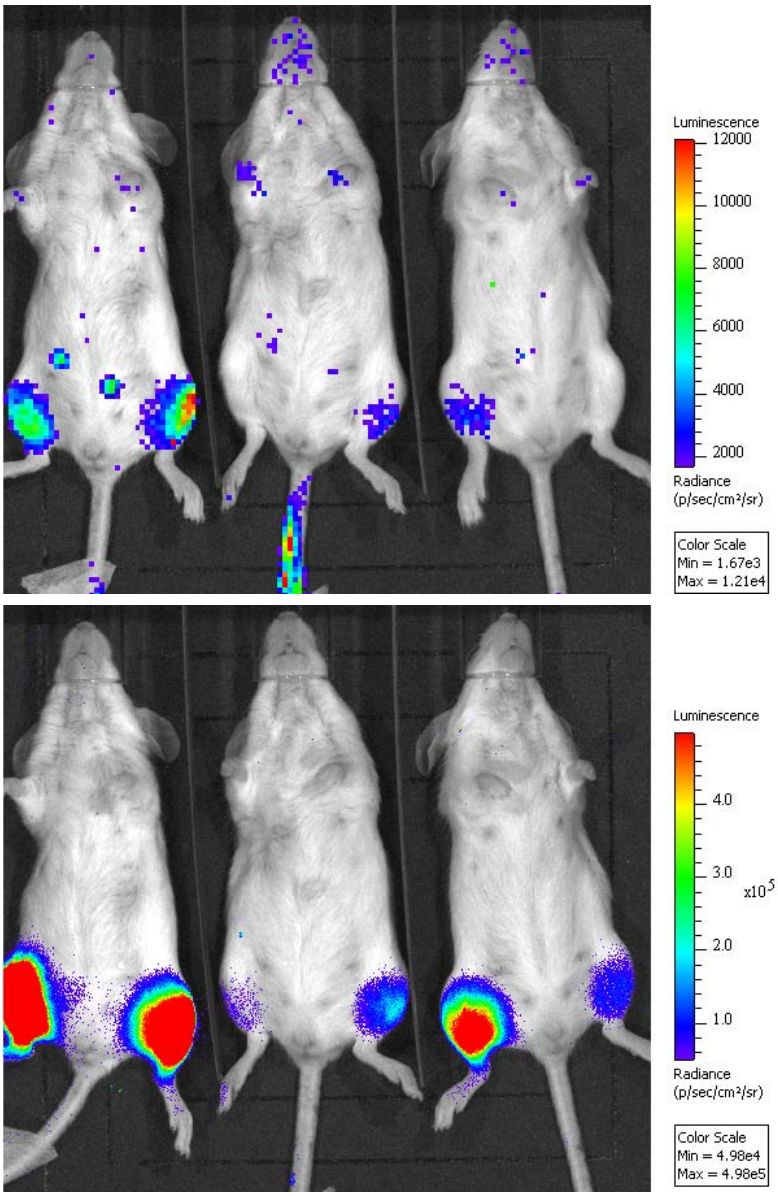


Figure 34: *In vivo* imagery of mice treated with siRNA-NPPRV1 (inside the green zone) or with an irrelevant siRNA. All mice were injected in the right and left legs with psiRNA-Fluc (30 µg/60 µl) and pGL4.51 (10 µg/60 µl), respectively. They were imaged at different time points after plasmid injections. The picture (a) shows the results at day 3 post-injection and the histogram (b) shows average total photons (p/s/cm²) measured in each group for the two siRNAs administered at different days after plasmid injection.

For the normalization of the signals, we selected a second bioluminescent marker. Although, it is possible to use a fluorescent marker in the IVIS-Lumina bioimaging system, in our hands, fluorescence signals were hardly detected from the TA muscle compared to the Firefly luciferase-2 gene (data not shown). The Renilla reporter gene was therefore evaluated. Three mice were co-injected with the two reporter genes in both legs and sequentially imaged, first after IV injection of Coelenterazine h and then after IP injection with D-Luciferin. Results are shown in Figure 35. Renilla luciferase was found to be 1.5 to 2 $\log_{10}$ less bright than Firefly luciferase-2, which is an expected result since the former is known to give less signal. For both signals, we could reproduce the variable success of intramuscular injections of the plasmid vectors. However, the dot plot shows a good correlation between the two markers. In conclusion to this trial, it appears that the results obtained from both legs of mouse 1, the left leg of mouse 2 and the right leg of mouse 3 could be exploited in a siRNA *in vivo* assay since normalization can be envisaged.

(a)



(b)

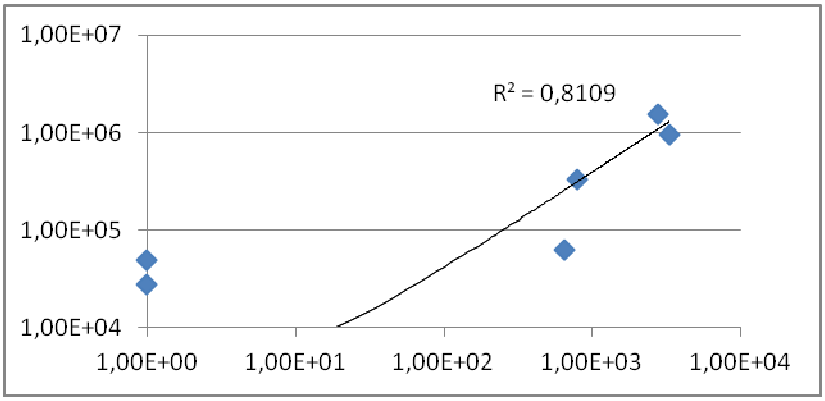


Figure 35: *In vivo* measurement of the co-expression of Renilla and Firefly luciferase genes. (a) Top picture shows Renilla activity, 4 minutes after IV injection of Coelenterazine h and on bottom picture the Firefly luciferase-2 activity, 10 minutes after IP injection of D-Luciferin. (b) The dot plot illustrates the correlation between the two signals.

GENERAL DISCUSSION

The viruses belonging to the *Morbillivirus* genus cause devastating diseases affecting humans, marine mammals, domestic and wild canines, small ruminants and large ruminants. They are emerging or re-emerging pathogens because of their high contagiosity and the difficulty to achieve a high enough immunity cover to prevent the appearance of epidemics. In spite of the availability of an effective vaccine, more than 30 million cases of acute measles with approximately 345,000 cases of infant deaths are annually reported [42]. For the control of measles, 95% of the population should ideally be vaccinated to avoid any new outbreak. But this objective is extremely difficult to achieve, even in Europe, because a small proportion of children is still not vaccinated or do not develop a sufficient immunity. Although effective vaccines against canine distemper are also available, a resurgence of the disease has been reported even in vaccinated dogs [49, 50]. After massive international efforts, rinderpest has been eradicated worldwide and the world will be declared rinderpest free by 2011 [38]. Eradication of rinderpest has brought PPR to the limelight, highlighting the need for its control. Although an efficacious vaccine is available and the fact that the disease hits the economically most vulnerable sections of world population [65], there is no international program for eradication of PPR.

The need for antiviral treatments concerns more particularly the diseases for which there are no efficacious vaccines. However, it is now possible to foresee antiviral drugs as an alternative or a complementary line of defence against infections. Thus, an RNAi based antiviral therapy against PPR may prove economically useful for control of epidemics not only if used alone but also along with vaccination. The vaccination against PPR is actually resorted to as an emergency measure in case of an epidemic. However, only the healthy animals can be vaccinated and it takes around a week for the development of the protection. In case of a PPR outbreak, use of an effective antiviral therapy along with ring vaccination in and around the disease pockets could help not only to control the epidemic but also to prevent extensive economic losses.

In a recent past, there has been a spectacular expansion of the comprehension of the molecular mechanisms of the viral cycles. Further, the efforts to contain the sudden rise of HIV have lead to the development of numerous antiviral molecules whose number has increased tenfold over the last ten years. Strategies developed to counter viral diseases have identified new molecular targets. Interfering RNA constitute an interesting trail to follow because of the *in vitro* selective degradation of the viral RNA renders them highly specific and effective in their action. However, even after more than a decade after its discovery, RNAi therapeutics are still

hampered by the challenge of *in vivo* delivery. Although siRNAs have been successfully delivered locally, for instance for treatment of age-related macular degeneration (AMD) [371], systemic delivery has proven more difficult. Majority of the current clinical trials for siRNA based therapies are for local administration while relatively few systemic delivery systems have entered clinical trials and almost all are still in Phase-I (Tab. 5) [372]. In this context, since PPR is an acute systemic infection, it is an extremely valuable model for systemic siRNA delivery with implications for treatment of measles in humans.

Table 5: Current development stage for siRNA therapeutics (according to Sliva and Schnierle., 2010) [373].

Disease	Mode of administration	Status	Company
Age-related macular degeneration (AMD)	Topical	Phase II	Allergan
Respiratory syncytial virus (RSV)	Local/direct	Phase II	Alnylam
Liver cancer (HCC and others)	Systemic	Phase I	Alnylam
Hepatitis B Virus (HBV)	Systemic	Phase I	Nucleonics
Solid tumors	Systemic/local	Phase I	Calando Silence Therapeutics AG
Acute renal failure	Systemic	Phase I	Quark Pharmaceuticals / Pfizer
Diabetic macular edema	Topical	Phase II	Silence/Quark/Pfizer
Metastatic melanoma	Local/direct	Phase I	Duke University
Pachyonychia congenita	Topical	Phase Ia/b	Transderm
High cholesterol	Systemic	Phase I	Tekmira Pharmaceuticals Corporation
Asthma	Systemic	Phase II	ZaBeCor Pharmaceuticals
HIV	Direct	Phase I/II	Benitec / City of Hope

The silencing capacity of the siRNA developed in CIRAD was achieved *in vitro* using delivery systems like liposomes and adenovirus. In the course of this study, the *in-vivo* phase was foreseen using the non viral in comparison to the viral vector systems. However for delivery of large quantities of siRNAs in the target species of PPRV commercialised chemical vectors are extremely expensive. . A in-house cationic lipid based formulation was therefore developed and used for *in vivo* delivery of siRNA NPPRV1. Replication deficient adenoviral human type 5 (Ad5) vectors are commonly used in siRNA based genetic therapy because they do not have the ethical problem associated to insertional mutagenesis which precludes the use of the retroviruses. In addition, during the time of this work, a study reported the successful therapeutic effect of adenoviruses expressing shRNA *in vivo* against foot and mouth disease virus (FMDV) infection in guinea pigs and swine [23, 362]. This led us to use our Ad5 vector

rAd_NPPRV1^{shRNA} for *in vivo* delivery of shRNA NPPRV1. Although the incidence of oculo-nasal discharges and stomatitis appeared to be reduced for animals treated with liposomes, the diarrhoea and pyrexia were in contrast increased and in any case, the differences were statistically significant. One of the possible reasons for this, was the insufficient relative siRNA/lipids concentration administrated to the animals resulting from the necessity to achieve acceptable injectable doses. Indeed, the use of diluted siRNA and lipid solutions for complex formation as were used for *in vitro* study, would have resulted in large volumes for intravenous injection which would not be feasible for injecting goats. However, while trying to use more concentrated siRNA and liposome solutions for achieving final injectable doses, a problem of precipitation occurred. Then, the siRNA/lipids ratio of had to be reduced by three for complex formation to avoid precipitation. This siRNA/lipids ratio tested *in vivo* was later on tested *in vitro* and was found to be less efficacious in PPRV CPE inhibition *in vitro*.

The lack of adenovirus effect *in vivo* could probably be due to differences in the tropism of the two viruses. While morbilliviruses, are primarily lymphotropic and secondarily epitheliotropic [61, 124, 132], lymphatic tissues are not transduced efficiently by adenovirus type 5 vectors [354] and are not correctly transfected by liposomes as well [374]. Whether the *in vivo* transfection or transduction of the epithelial tissues alone, with insufficient transfection/transduction of lymphatic tissues, can suffice for having a therapeutic effect cannot be confirmed. Similarly, Chen *et al*., could protect swine from a major clinical disease through adenoviral vectors targeting FMDV, however they incriminated the different tissue distribution of rAd5 and FMDV as responsible for the limited inhibition of FMDV infection *in vivo* [23]. For effective delivery of siRNA/shRNA against PPRV, the transfection/transduction of lymphatic tissues is desirable for improved therapeutic effect. As perspectives, ligands targeting lymphocytes, could be attached to metabolically biotinylated adenoviral [375], baculoviral vectors, liposomes and CPPs for improving transduction/transfection of the target tissues. These modified viral and chemical vectors could be tested *in vitro* on goat PBMCs. However for initial screening of unmodified vectors *in vitro*, the Vero cells remain ideal as they lack genes for interferon (IFN), while if lymphocytes or other cell lines are used the effects of IFN may over shadow the contribution of RNAi upon PPRV replication [376-378]. RNAi experiments in goats create several practical difficulties. In our *in vivo* experiment, we could not test several doses for adenoviral vector *in vivo* as it would have required large number of goats; therefore in future *in vivo* dose optimization is still needed. The results of Chen *et al*., indicated that all three swine receiving a low adenoviral dose (4 X 10^9 PFU) were completely protected against FMDV challenge.

However, the antiviral effect was somewhat impaired in the animals treated with a high dose of adenoviruses (8 X 10^9 PFU) whereby only one animal was protected [23]. They suggested that this may have been due to inhibitory effect of adenoviral VA1 non-coding RNA, which they think should be removed in future for more efficient delivery [23].

Since the first attempt at *in vivo* delivery of siRNA did not give the expected results, it entailed us to search for more efficient viral or non-viral vectors which could be possibly used for future siRNA/shRNA delivery *in vivo*. For this purpose, recombinant adenovirus and baculovirus vectors expressing short hairpin RNA (shRNA) against the PPR virus (PPRV) were prepared and compared for efficiency *in vitro*. We found a lower efficiency of the adenovirus against PPRV replication compared to previous studies in which other pathogens were targeted [362]. In contrast, the recombinant baculovirus tested in this study proved to be more efficient than adenovirus vector when used at same MOIs. A reduction of 2.19 $\log_{10}$ on PPRV replication was observed which is more than 1 $\log_{10}$ higher than other comparable studies involving baculoviral vectors [335, 364]. However, the reduction of NPPRV protein expression by 73% and PPRV progeny titre by 2.19 $\log_{10}$ with rBac_NPPRV1^{shRNA} was lower than the one previously observed by our group when the siRNA were delivered to cell cultures by Lipofectamine 2000™. The latter reduced the PPRV nucleoprotein expression by 90% and progeny titre by 3-4 $\log_{10}$ [350]. Although recombinant baculoviruses are easier and cheaper to produce in high quantities than liposomes and do not have deleterious effects up on mammalian cells even at very high MOIs [339], they are susceptible to complement inactivation [336]. Chemical or genetic modifications, however, has been reported to be able to overcome this problem [337, 338]. In addition to baculovirus, we also investigated two new peptides (CPPs) named PF6 and PF14 for siRNA delivery. Transfection of siRNA NPPRV1 with PF6 at 100 nM resulted in the silencing of viral protein expression by up to 99% which was higher than the inhibition achieved with Lipofectamine 2000™ (90%, [350]). In contrast, the PF14 was less efficient for the transfection of siRNA NPPRV1. Transfection could be improved by using higher siRNA/PF14 ratio but still it never reached efficacy of PF6. Unfortunately, the availability of PF14 was limited and we could not increase the ratio siRNA/PF14 as required. This was a pity since our results tended to show that PF14 was less prone to the negative effect of the presence of serum in the environment of the cells where had to be delivered the siRNA. In provisional conclusion, we could not discriminate clearly the suitability of two CPPs for future studies *in vivo*. This will require new *in vitro* trials or to test the two CPPs *in vivo* with the recombinant baculovirus. However, such an *in vivo* test is not feasible in the target species since it requires many large animals and large quantity of

expensive siRNA. Therefore, development of a small animal model to assess the *in vivo* performance of different delivery systems became an absolute necessity in the course of this work.

To decrease the technical leap between the *in vitro* and *in vivo* evaluation and also improve the ethical, bio-security and economic aspects of the *in vivo* experiments the mouse model was investigated. From a technical point of view, a BALB/c mouse is more than 1000 times smaller than a goat and thus requires much lower quantities of siRNAs and vectors for experiments. Furthermore, the RNAi experiments with virulent PPRV require high containment animal facilities which are not available at CIRAD. Although PPRV infection of mice could be possible at the high containment laboratory of CIRAD, normal mice are resistant to PPRV infection. We tried to identify a susceptible mouse that could be infected by PRRV. IFN knockout mice were tested because they were recently shown to be highly susceptible to another ruminant virus, an orbivirus responsible of bluetongue disease [379]. However, PPRV challenge on these mice was unsuccessful since neither clinical signs nor viremia was detected. This failure is probably due to inability of PPRV to use mouse SLAM or CD150 receptors. To continue in this direction, the production of transgenic mice expressing sheep SLAM receptor will be required. Such a model was successfully developed for measles virus [380]. In the absence of a model for PPRV, a non-infectious strategy was developed instead to enable *in vivo* assessment of siRNA delivery. For this purpose, a luciferase expressing reporter plasmid having siRNA NPPRV1 target sequence was developed. Aim was to inject this construct into the mouse muscle and deliver siRNA via various vectors and measure effect of siRNA on luciferase expression by CDD camera. The siRNA NPPRV1 could knock down luciferase expression by psiRNA-Fluc *in vitro*, thus illustrating that the siRNA target sequence placed upstream of the Firefly luciferase 2 reporter gene was efficaciously targeted by our siRNA. The amount of psiRNA-Fluc required for injection into the mouse tibialis anterior (TA) muscle was determined for best *in vivo* imagery by CDD camera. Next, a liposome-based formulation developed by a private company was tested against our psiRNA-Fluc reporter system. However, luciferase expression was found to be inconsistent between legs and mice with some non-responding individuals. The variability was assumed to arise from the plasmid injection and not from the substrate bioavailability after intraperitoneal injection (performed in two steps at different sites to avoid possible failures of intraperitoneal injections). Owing to this variability, the difference observed between the treatment and control groups, considering the expression of psiRNA-Fluc, was not significant. In order to manage this variability, a double labeled system was introduced,

using Renilla luciferase as a second house-keeping bioluminescent reporter to check the quality of plasmid injections and to normalize the results of siRNA activity. For both signals, the variability in accuracy of intramuscular injection of plasmid vectors was observed, however, a good correlation was obtained between the two markers, thus allowing normalization. Since normalization could be achieved, it appears that this mouse model can now be used for testing of siRNA delivery *in vivo*. Use of this bioluminescent reporter based mouse model would allow us to test not only the various siRNA or shRNA expressing viral vector doses but also would enable us to test other parameters like vector toxicity. The ethical aspects are also considered in this model since the mice are not infected but only receive reporter plasmids by the intramuscular route followed by daily injections of luminescent substrates, either intravenously or intraperitoneally. All administrations are practiced under gas anesthesia. Therefore, the animals suffer less as compared to a PPRV infection in small ruminants.

CONSCLUSION AND PERSPECTIVES

A recombinant replication deficient adenovirus and a baculovirus expressing shRNA against nucleoprotein of PPRV were tested *in vitro*. This study showed that both recombinants can inhibit PPRV replication *in vitro*. However, the baculoviral vector was found to be more efficient. A CPP PF6 can deliver siRNA NPPRV1 effectively *in vitro* resulting in an almost complete inhibition of N gene expression by PPRV. Although PF14 has a lower transfection efficiency *in vitro* at the siRNA/PF14 ratio of 1/15, use of higher ratios may improve transfection. Furthermore, it is relatively serum resistant compared to PF6. Whether these vectors which have been found to be efficient *in vitro*, would equally be effective in siRNA delivery *in vivo* for inhibition of PPRV replication, needs to be elucidated.

Since the systemic delivery of siRNA is the key issue for future therapeutic application, we have investigated a strategy based on the use of a non-infectious mouse model and a dynamic follow up of siRNA treatment by live imaging. We show in this work that it is possible to measure and standardize the expression of a bioluminescent reporter gene and thus, to quantify a down-regulation of such gene. The work is on-going to calibrate this experiment and then siRNA delivery will be tested again circumventing the initial problems of variability that result from inconsistent plasmid injections. This model will be very useful for comparing various vectors for systemic delivery of siRNAs. At the moment, in addition to the Aonys system by Medesis Pharma, our recombinant baculovirus and the CPP PF6 or PF14, described in the part 2, are promising candidates.

Once the best candidates will have been identified in this non infectious model, the objective will be to confirm our observation in an infectious mouse model. Since mice are naturally resistant to PPRV, our approach will be based on the generation of a transgenic mouse expressing the sheep SLAM receptor for PPRV and being deficient for interferon type I responses (double knock-out mouse for interferon type I receptor: IFNAR-/-). A similar model (e.g. IFNAR-/- mice expressing human SLAM receptor for the measles virus) was developed for measles virus [365]. The idea is to adapt this model to PPRV. Once established, we will be able to test the RNA interference in mice *in vivo*: the inhibitory effect of siRNA will be measured in terms of reduction of clinical signs or mortality. In addition, the laboratory is also developing a bioluminescent PPR virus by reverse genetics. The final goal will be to test the control of PPRV replication by siRNA through bioluminescence imagery.

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